Knockdown of CRPV E6 and E7 Oncogenes Using Lipopolyplexes as Transfecting Reagents

Dissertation

zur

Erlangung des Doktorgrades

der Naturwissenschaften

(Dr.rer.nat.)

dem

Fachbereich Pharmazie

der Philipps-Universität Marburg

vorgelegt von

Uzma Ali

aus **Jhang, Pakistan**

Marburg/Lahn 2020

Erstgutachter: **Prof. Dr. Udo, Bakowsky**

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Eingereicht am **19.10.2020**

Tag der mündlichen Prüfung am **09.12.2020**

Hochschulkennziffer: 1180

Knockdown of CRPV E6 and E7 Oncogenes Using Lipopolyplexes as Transfecting Reagents

Thesis

Submitted in the fulfilment of the requirements of degree of

Doctor of Natural Sciences (Dr.rer.nat)

equivalent to

Doctor of Philosophy (Ph.D.)

To

The Faculty of Pharmacy,

University of Marburg.

by

Uzma Ali

from **Jhang, Pakistan**

Marburg/Lahn 2020

First Supervisor: **Prof. Dr. Udo, Bakowsky**

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Date of Submission **19th October 2020**

Defense date **9th December 2020**

Hochschulkennziffer: 1180

E I D E S S T A T T L I C H E E R K L Ä R U N G

Ich versichere, dass ich meine Dissertation

"Knockdown of CRPV E6 and E7 Oncogenes Using Lipopolyplexes as Transfecting Reagents”

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Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 19.10.2020

.....

(**Uzma Ali**)

A U T H O R'S D E C L A R A T I O N

I declare that this thesis titled

"Knockdown of CRPV E6 and E7 Oncogenes Using Lipopolyplexes as Transfecting Reagents"

has been written entirely by myself and is a record of work performed by myself. The research was carried out at the Institut für Pharmazeutische Technologie und Biopharmazie, University of Marburg, at the campus Ketzerbach 63 (old location), Robert-Koch-Straße. 4 (new location) and Universitätsklinikum Gießen und Marburg under the supervision of Professor Udo Bakowsky and Professor Robert Mandic.

This thesis has not been submitted in any form elsewhere for a higher degree.

Marburg, 19th October, 2020

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(**Uzma Ali**)

A C K N O W L E D G E M E N T S

This research project was proposed by Prof. Dr. Udo Bakowsky in mutual collaboration with Prof. Dr. Robert Mandic and was carried out at Institute of Pharmacy and Biopharmaceutics, Faculty of Pharmacy Philipps University Marburg and Universitätsklinikum Gießen und Marburg GmbH, Philipps-Universität Marburg from 2016-2020.

I would like to offer a special word of gratitude to Prof. Dr. Udo Bakowsky for his patience, enthusiasm, encouragement, wise advice and support throughout the course of my doctoral studies. I am fully indebted to Prof. Dr. Robert Mandic for his brilliant ideas, at length discussions and motivation which made it possible to complete this research project. Prof. Dr. Michael Bette has been a great mentor throughout this research journey. I appreciate his commitment, participation, logic based input and above all his optimism.

A special note of thanks to Dr. Shashank Pinnapireddy for his guidance, patience and immense support. My deepest gratitude for Ms. Eva Maria Mohr, Ms**.** Roswitha Peldszus and Ms. Grazyna Sadowski for their technical support and guidance at every step. These ladies are the most patient, motivated and inspirational. A very special thanks to Ms. Julia Michaelis for her positive vibes, encouraging words, patience, therapeutic voice and for being the most calming human I ever came across. I would like to express my appreciation to Dr. Konrad Engelhardt for his positive feedback and great help.

A big thank you to all my family and friends-Aamna, Bushra and Ghazala for bearing with me through the entire length of my doctoral studies, their constant uplifting words of encouragement and unconditional love. I owe immense gratitude to my husband, Waseem, my kids, Manahil and Meeral, my Ammi and Abbu and my siblings, Ali, Haider and Asma for always being there with me through thick and thin.

I dedicate this thesis to my late father, Ahmad Ali Sajid!

Die vorliegende Arbeit entstand auf Anregung und unter Leitung von

Herrn Prof. Dr. Udo Bakowsky
und
Herrn Prof. Dr. Robert Mandic

am Institut für Pharmazeutische Technologie und Biopharmazie der

Philipps-Universität Marburg

Universitätsklinikum Gießen und Marburg GmbH, Philipps-Universität Marburg

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Part I: Introduction

1.1 Gene therapy

Gene therapy has been widely associated for the treatment of a wide range of gene anomaly associated diseases such as cancer, autoimmune diseases and neurodegenerative diseases [1]. Gene therapy is based on introducing the genetic cargo into the target tissues and thus inhibiting or altering the expression of endogenous genes in order to prevent the spread of the disease. The progression in the field of cancer gene therapy has revealed abundant potential for various forms of treatment. Various in vivo and in vitro therapetic strategies have shown remarkable results such as in case of lung cancer, gene therapy application to create cancer vaccines, introducing viruses into tumors in order to cause lysis followed by cell death or instigating genes into cancer cells causing either cell death or restoration of normal cellular functions and phenotype [2].

The initial hype and enthusiasm for this revolutionary field of gene therapy is however taken a turn towards more cautious therapeutic approaches after realisation of the associated risks and setbacks such as death of a patient taking part in a dose escalation gene therapy trial in 1999 [3]. Although this incident was not related to cancer treatment but a metabolic disorder (ornithine transcarbamylase deficiency), this led to re-evaluation of all gene therapy trials for safety. A lot of new practices were incorporated and thousands of patients took part in clinical trials of cancer gene therapy and showed eminently fewer adverse effects as compared to the side effects observed with traditional chemotherapy [4].

Gene delivery vehicles play a major part to initiate a potential adverse effect and most of these adverse effects are observed because of viral vectors such as immunogenicity and higher cytotoxicity. The first reported gene therapy clinical trial fatality was related to inflammatory reaction because of Adenovirus which was used as a viral vector for gene delivery [5]. Thus non-viral vectors are deemed more safe to use although there are challenges for efficient transfection because of intracellular and extracellular barriers. However, lesser pathogenicity, ease of production and cost-effictiveness are the characteristics which make non-viral vectors better candidates for gene delivery than viral vectors [6]. Non-viral vectors for gene therapy include naked plasmids, chemical based and particle based. The major aspects which need to be thoroughly investigated before selection of a non-viral vector for gene therapy are: the effectiveness of vector transport and offloading of nucleic cargo into target cells, tenacity,

immune response, activity, regulative concerns, ethical considerations and commercialization [7, 8].

1.2 Nanocarriers for gene therapy

Nanoparticles are believed to be the most promising gene delivery vehicles because of their shape, tunable size, surface characteristics and biological behavior [9]. Nanocarriers possess cationic surfaces which provide facilitation to encapsulate anionic nucleic acids cargo for intracellular delivery [10]. These cationic nanocarriers include cationic liposomes, micelles, polymers, hydrospheres and hydrogels etc. which have been investigated extensively for their potential as gene delivery systems. However, most of clinical trials until present involve cationic liposomes as gene delivery vehicles for their efficacy and safety for plasmids as well as small interfering RNA [11]. Among recent studies on nanocarriers, lipopolyplex has emerged as a very promising non-viral vector for gene therapy because of its exceptional colloidal sustainability, marginal cytotoxicity and exceeding transfection efficiency [12]. Lipopolyplex is designed as a second generation non-viral nucleic acid vector comprised of liposome and polyplex following the first generation liposome-DNA complex [12, 13].

Lipopolyplex is composed of two major entities, liposomes and polyplexes.

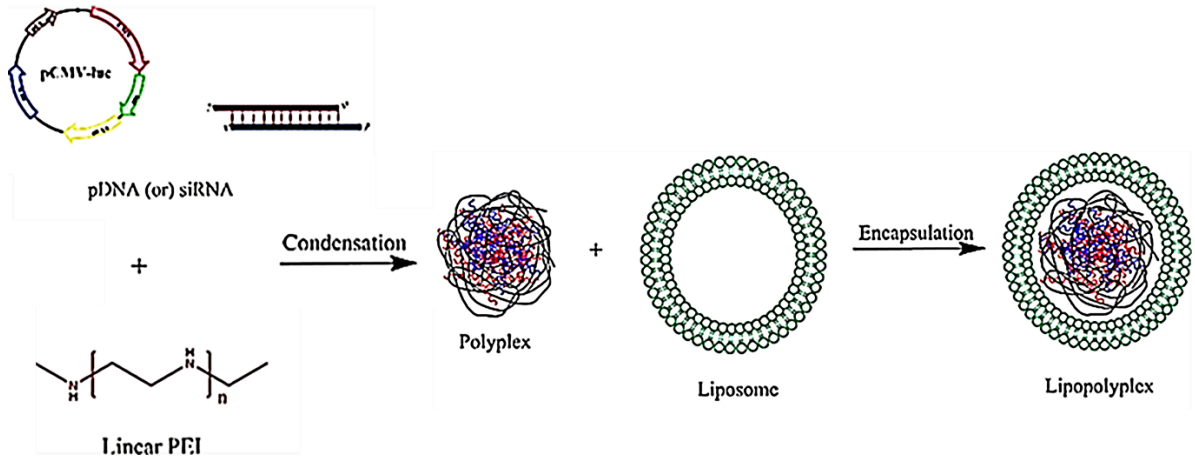

Figure 1: *A graphical abstract of lipopolyplex formation* [14]

In previous studies, polyethylenimine (PEI) has been found an efficient nucleic acid vehicle both *in vitro* as well as *in vivo* [15] because of its high nucleic acids compaction ability and innate endosomolytic capacity [16]. Polyplexes are the complexes or composite aggregates of nucleic acids with cationic polymers, which in our studies are plasmid DNA or siRNA and lPEI [14]. The lPEI is deacylated PEI which is an improved version of linear PEI because after deacylation, its transfection efficiency is remarkably increased and cytotoxicity is significantly decreased because of lack of primary amines which are frequently present in branched PEI causing high charge density leading to compromised cell wall integrity causing cellular necrosis [17]. These polyplexes, containing nucleic acid and lPEI, often induce immune responses because of their overall cationic nature [18]. To address this problem, these polyplexes were wrapped with liposomes composed of DPPC, DOPE and cholesterol.

DPPC: Dipalmitoylphosphatidylcholine contains a fatty acid polar phosphate head group along with a nonpolar chain of fatty acid. DPPC molecules can arrange according to polar and nonpolar interactions with a phospholipid bilayer forming spherical vesicles [19].
DOPE: Dioleoylphosphoethanolamine is a non-bilayer synthetic, unsaturated, conical phospholipid. DOPE arranges into hexagonal inverted phase because of its negative spontaneous curvature [20]. Cholesterol: Cholesterol is a single polar hydroxyl group lipophilic molecule. Cholesterol stabilises liposomes and bio-membranes by providing mechanical compactness and elasticity. Moreover, it enhances phosphatidylcholine vesicle resistance and deters vesicle aggregation [21].
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1.3 siRNA mediated gene therapy for cancer treatment

The phenomenon of RNA interference in animals was first reported in 1998 when Fire et al found that a complementary double stranded RNA can act as a gene silencer in Caenorhabditis elegans [22]. Initial observation of gene silencing in plants was reported in early 90's and expressed the same pattern of gene silencing by transcript-dependent endonucleolytic breakdown of mRNA [23]. RNA silencing is defined as post-transcriptional inhibition of gene expression by silencing mRNA [24]. RNA interference is believed to be a promising substitue for convetional cancer therapies being less cytotoxic than traditionally used chemotherapy. siRNAs have been investigated for the cure of various human pathologies such as ocular

diseases, genetic abnormalities, cardiovascular conditions, viral infections as well as cancers. The potentiality to target any gene is the most appealing characteristic of siRNA treatment approach when other therapeutic approaches are not suitable for therapy [25].

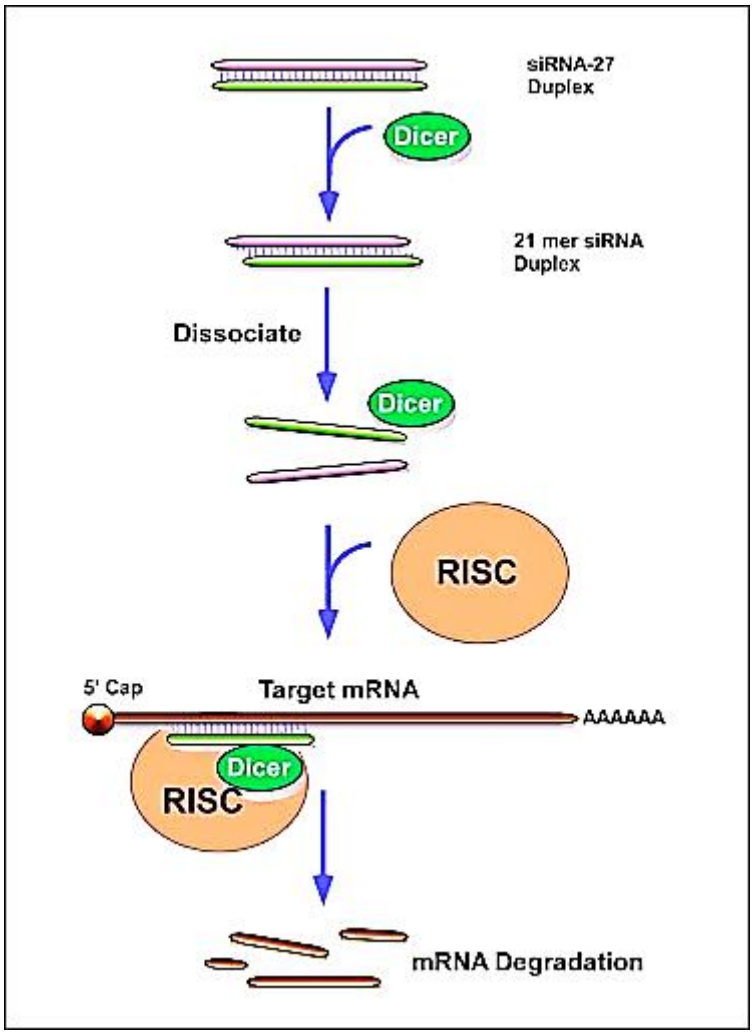

Figure 2*: Graphical representation of siRNA induced mRNA inhibition*

Gene silencing by siRNA knockdown has revolutionized molecular and cell biology studies. The basic mechanism of action of siRNA mediated gene silencing involves instigation of impairment in complimentary strand of mRNA by neutralizing it thus rendering its use as a translation template [26, 27]. This is achieved by exogenous introduction of small interfering RNAs into the cell where these are dealt with RISC (RNA inducing silencing complex) which

helps a template of siRNA to locate and target the complementary mRNA resulting in the cleavage of mRNA by a ribonuclease. Thus mRNA becomes unattainable for the translation process and thereby causes inhibition of protein expression [28-31]. siRNA has the potential to treat not only cancers but also infectious diseases and disorders that originate from genetic disorders or mutations. siRNA mediated gene knockdown has become a standard tool for molecular targeting therapy [27, 32].

1.4 Transfection of siRNA into cells

siRNA can be introduced into eukaryotic cells either by a viral or non-viral delivery vehicle. Viral transfection, also known as transduction, has been widely used in clinical research because of high transfection efficiency and persistent transgene expressions [33]. Most common viruses used for this purpose are Lentivirus, Adenovirus, Retrovirus and Adeno-associated virus etc. However the drawbacks such as mutagenesis, cytopathic changes and high costs of using viral transfection limit their use [34].

Non-viral techniques of transfection include either chemical methods usually invloving nanoparticles, lipofection, polymeric carriers of genetic material: polyplexes etc. [35] or physical methods such as direct micro injection, electroporation, magnetofection, cell squeezing etc. Transfection of genetic material into cells can be achieved with the goal of transient or stable transfection [36-38].

Stable transfection results in the integration of transfected genetic material into the host genome and also inheritance of this genetic material by the daughter cells, thus causing that cell line to express altered characteristics of introduced genetic material permanently.
Transient Transfection, on the other hand, results in temporary introduction of foreign genetic material into a cell, where it does not become part of the host genome and is diluted out after cell division over time resulting in the loss of expression of the transfected gene [39].

1.5 Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) affects more than 800,000 patients every year, representing the 6th most common cancer world-wide [40, 41]. Standard treatment strategies for HNSCC involve surgery, radiation, chemotherapy or a combination of all these but these options are still incapable of decreasing the mortality rate significantly [42]. To improve rather scant outcome of therapies which are in practice for HNSCC, molecular-targeting approaches have given a new direction to further therapeutic options [43]. Molecular therapies against cancers focus on targeting specific molecules responsible for cancer development, advancement and metastasis [44] instead of assaulting all cells indistinctively (as in conventional chemotherapy).

All of neoplastic diseases emerge from five mutual traits [45] which are:
Impeccable continuation of proliferative signalling
Eluding growth suppressors
Combating cell death
Facilitating replicative immortality
Commencing angiogenesis, boosting up invasion and metastasis

Targeted molecular therapy techniques against cancers specifically prey one or more of the regulatory factors controlling above mentioned mechanisms which are responsible for various stages of a tumour formation. For *in vivo* testing of anti-viral therapies for treatment of HPV induced carcinomas such as HNSCC, the only available models are immunocompromised mice and human tissue xenografts [46]. The basic drawback of this model system is the lack of natural host system during development of papilloma [47].

1.6 Parallel pattern of HPV and CRPV induced carcinomas

As this study is based on the comparison of parallel efficacies of HPV E6 and E7 oncoproteins with CRPV E6 and E7 oncoproteins, so it is important to understand the role of former mentioned genes to grasp the mode of action of the CRPV oncogenes.
Two major types of oncoproteins are encoded by HPV, E6 and E7, which are directly accountable for HPV carcinome progression [48, 49]. These HPV oncogenes work side by

side to target various cellular pathways responsible for cell cycle regulation, apoptosis and controlling mechanisms of cell polarity [50-52].

1.7 Role of HPV E6 and E7 oncogenes

Human Papilloma Virus has five species: (α) alpha, (β) beta, (γ) gamma, mu and nu papilloma viruses [53]. Among these, α and β are found the most intensively infectious types. Alpha HPV types are divided into low risk, LR, and high risk, HR types [54, 55]. Low risk α type HPV-6 and 11 usually cause benign lesions whereas high risk α type HPV-16 and 18 are responsible for malignant cancers in ano-genital and head and neck areas [56]. In case of HPV associated head and neck carcinomas, which assault oropharynx, HPV-16 is the most frequent cause worldwide [57, 58].

Alpha-HPV species infect the mucosal epithelial layer by infiltrating through micro-bruises or mild lesions. As virus cannot reproduce by itself, so it exploits cellular epithelial differentiation processes to replicate and in this process HPV E6 and E7 play a vital role. These viral genes sabotage cell cycle regulation and apoptosis in cells of the basal layers that results in very high viral copy number and tumor formation [59, 60]. In case of some persistent infections, viral DNA somehow integrates into host genome, causing loss of viral proliferation ability and most of viral genes except E6 and E7 oncogenes which are uncontrollably expressed and cause immortalization of cells resulting in the development of malignancies [61, 62].

HPV E6 and E7 oncogenes, when expressed in combination, cause immortalization of cell lines derived from tumors as E6 compromises cellular survival pathways while E7 promotes cells proliferation [63]. However, when these genes are expressed individually, they do not result in dramatic effects on immortalization of cells and fail to induce neoplasm or carcinoma in spite of the fact that E6 causes elevated copies of centrosomes and abolishes traceable levels of p53 protein whereas E7 results in higher number of centrosomes and elevated proliferation [64]. In case of HNSCC, E7 oncoprotein is found to be the main transforming factor while E6 is considered to play a vital role in later stages of tumor development [65].

1.8 Role of CRPV E6 and E7 oncogenes

CRPV E6 and E7, without any other co-factors, are found to immortalize rabbit keratinocytes *in vitro* and this ability to proliferate cells uncontrollably is also a characteristic of HPV E6 and E7 oncogenes which is believed to be a prerequisite in the formation of all HPV cancers [66]. Cottontail rabbit papilloma virus animal model is used extensively for therapeutic investigations of HPV associated carcinomas because both of these models have parallel efficacy and resemblance in papilloma virus-induced cancers progression [67].

Various reports have been published about downregulation of HPV E6 and E7 ocnogenes either directly, by siRNA silencing or indirectly, by inhibition of various cancer markers and their consequent effect on tumor growth and cell proliferation markers [68-70]. These studies have well established the role of HPV E6 and E7 genes to offset p53 and Rb tumor suppressor pathways respectively. Interestingly, no current information is available on siRNA silencing of CRPV E6 and E7 oncogenes. CRPV generates three proteins, CRPV LE6, SE6 and CRPV E7 which are responsible for anchor independent proliferation in immortalized cell lines [71].

CRPV E6 proteins, unlike HPV E6, have been found to be unable to bind with and thus inhibit p53 in mouse embryonic carcinoma cells [72] which indicates that in spite of having almost similar charactersitics as HPV E6, CRPV E6 proteins also show some contrasting traits as it is important to take into consideration both similar as well as opposite factors while comparing two parallel treatment strategies. However, CRPV E7 protein has analogous characteristics as HPV16 E7 such as binding to pRb, inhibition of complex formation between pRb and the E2F transcription factor, transactivation of adenovirus E2 promotor and facilitating the cellular transformation in rodent fibroblasts as determined by growth in soft agar [73].

CRPV E7 is also shown to immortalize rabbit keratinocytes in the absence of mitogenic stimuli by decreasing rabbit $p19^{ARF}$ which inhibits ubiquitin ligase activity resulting in an increase in the p53 protein half-life. However, this accomplice for p53 does not ffect on cells proliferation. CRPV E6 does not bind to p53 unlike high risk HPV E6 proteins and even after proteasomal inhibition, p53 remains active on transcriptional level and induces p21 [56].
How CRPV and low risk cutaneous and genital HPV carcinomas, inspite of their inability

of E6 proteins to bind to p53, still manage to escape p53-mediated growth arrest, is not answered yet [56].

Several groups have discussed the importance of CRPV E1, E2, E6 and E7 proteins to induce papilloma formation in rabbits. So it is well established that all of these CRPV early genes provide possible targets for molecular targeting strategies to treat CRPV associated carcinomas [74, 75].

1.9 *In-vitro* system for therapeutic evaluation of Papilloma virus induced cancers

The parallel efficacy of therapeutic strategies to treat CRPV induced carcinomas in cottontail rabbit versus treatments used for HPV associated cancers, has been previously established in different clinical settings [76, 77]. The CRPV rabbit model system provides a remarkable foundation to investigate a wide range of antiviral therapies *in vivo*.

The analogous pattern of growth of CRPV-induced carcinoma and HPV associated cancers makes the cottontail rabbit model a promising candidate to explore various *in vivo* treatments [78, 79]. However, the major limitation of this model is the variability in immunocompetent rabbits to respond to CRPV infection and treatments thus increasing the cost of investigating therapeutic compounds quite significantly [76]. Hence an *in vitro* system is essential to study different therapeutic approaches for the treatment of papilloma virus dependent carcinomas. Various publications reporting the establishment of $CRPV^{+}$ VX2 carcinoma cell lines have appeared previously over the years [80-83], however, none of these cell lines was available commercially for investigative purposes, so in a different study, we have developed a CRPV carcinoma derived VX2 cell line which has been characterized extensively and here we use this VX2 cell line to apply gene therapy techniques to treat CRPV carcinoma.

Transient Transfection, on the other hand, results in temporary introduction of foreign genetic material into a cell, where it does not become part of the host genome and is diluted out after cell division over time resulting in the loss of expression of the transfected gene [39]. In this study, we used transient transfection as a source to introduce genetic material or siRNA into

cells. Here lipopolyplexes were used as transfecting reagents.

1.10 Effect of stress inducing factors on oncogenic expressions

1.10.1 Serum starvation

Previous studies have shown cancer cells exhibiting resistance to serum starvation or nutrients depletion. For example, in case of breast cancer, Gli1, an activator of the hedgehog (Hh) signalling pathway and being involved in cell survival and cancer growth, is up-regulated in serum starved cancer cells [84].

In several studies, serum starved LoVo colon cancer cells showed alterations in ADMA level and also significant transcriptional fluctuations of genes involved in apoptosis, cell cycle, p53 signalling pathway and nucleic acids metabolism [85] .

Chinese Hamster lung fibroblasts, V79, are adherent cells and grow in monolayer in serum enriched medium but when exposed to serum starvation, start rounding up and cause upregulation of p53 and PKC-α proteins. An extended period of serum starvation (≥48h) may lead to cell detachment and death in v79 cells [86].

In human colorectal cancer cell lines such as HCT 116 and breast cancer cells cultured in scarce-nutrient situations express higher levels of p21, independent of any relation to p53, which causes p21 to save cells from serum depletion induced apoptosis and cell death [87].

1.10.2 Effect of cisplatin treatment on cancer cells

Serum starvation of non-cancerous cells causes growth arrest and indifferent response towards cisplatin toxicity whereas in case of cancer cells, mild effect on proliferation rate and significant susceptibility for cisplatin toxicity because of activation of ATM/Chk2/p53 signalling pathway is observed under serum deprivation conditions [88]. Cisplatin exposure alone can cause significant alterations in transcriptional levels of different genes such as GJA1 and TWIST1 involved in early development of cells and cell to cell communication. P53 levels are also found to be amplified after exposure to higher doses of cisplatin in both drug sensitive and drug resistant cancer cells [89].

In ovarian cancer cell lines, Cisplatin treatment has shown to cause down-regulation of MCTP1 and up-regulation of S100A3 and C4orf18 genes which offer major resistance to cancer cells against cisplatin. Cisplatin induced variations in expression of various genes can lead to therapeutic solutions involving targeting these differently expressed genes and reversing drug resistance in cancer cells [90].

1.10.3 Effect of radiation on cancer cells

Oncogenes exhibit a change in usual pattern of transcriptional levels after radiation in both normal tissues and cancer cells [91, 92]. When wild type C57BL/6 mice was exposed to high-photon based and charged particles radiation, magnified expression of 78 oncogenes was observed in liver tissues whereas 88 oncogenes were expressed differently in mouse spleen, lungs, brain, kidneys, liver as well as in small and large intestines post HZE ^{56}Fe particle radiation [93].

Other studies have demonstrated fluctuation of gene expression profiles after radiation, some of which depend upon type of cell line while few special genes are found to be associated with high resistance status of cancer cells [94, 95].

Aims and Objectives

Aims and Objectives

The primary purpose of this work was to determine the versatility of lipopolyplexes as non-viral vectors for gene therapy and establishing non-conventional techniques to evaluate their transfection potential in different cell lines. Another goal of this study was to fill the space between parallel therapeutic options regarding HPV and CRPV induced cancers as both of these carcinomas originate from closely related set of oncogenes. For this purpose, CRPV E6 and E7 oncogenes were targeted by siRNA and downregulation of their gene expressions was monitored by different scientific assays.

The key elements of this study include:

1. Preparation of pre-designed lipopolyplexes and affirmation of their physicochemical characteristics
2. Determination of transfection efficiency of lipopolyplexes in VX2 and COS-7 cells
3. Evaluation of cytotoxicity of lipopolyplexes in VX2 and COS-7 cells
4. Detection of CRPV E6 and E7 transcripts in VX2 cells
5. Knockdown of CRPV E6 and E7 oncogenes in VX2 cells by using siRNA loaded lipopolyplexes
6. Designing a parallel system for evaluation of siRNA efficiency to downregulate CRPV E6 and E7 oncogenes in the absence of a VX2 cell line
   -Transfection of GFP tagged CRPV E6 and E7 oncogenes in COS-7 cells followed by siRNA mediated knockdown
   -siRNA-mediated downregulation of CRPV E6 and E7 oncogenes in VX2 tumor suspension
7. A detailed account of limiting factors affecting gene therapy of CRPV carcinoma
   -Oncogenic expression shift of CRPV E6 and E7 under different *in vitro* conditions

Part II: Experimental

2.1 Materials and instruments

Instruments and Materials	Sources
0.2 µmol/L PES Syringe Filters	Whatman plc, Buckinghamshire, UK
0.2µmol/L non-Pyrogenic Filters	Schleicher and Schuell, Dassel, Germany
12-well plates; Nunclon Delta	Nunc GmbH and Co. KG., Wiesbaden, Germany
96-well Microtiter Plates; CytoOne®	Starlab International GmbH, Hamburg, Germany
Adhesive Plate Seals	Boehringer Mannheim GmbH, Mannheim, Germany
Amphotericin B	Biochrom, Berlin, Germany
Ampicillin	Sigma Aldrich, St. Louis, MO, USA
Ampicillin	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Autoradiography Cassette	Agfa, Köln, Germany
Bath Sonicator; Transonic Digital S	Elma Schmidbauer GmbH, Singen, Germany
BD FACS Calibur Flow Cytometer	Becton Dickinson, Heidelberg
Bradford Spectrophotometer	Hamburg, Germany
Cell Culture Hood (work bench)	Heraeus, Hanau
Cell Culture Lysis Reagent	Promega GmbH, Mannheim, Germany
Centrifuge 5430R	Naumburg, Germany
Centrifuge 5920R	Naumburg, Germany
Chloroform	Roth, Kalsruhe, Germany
Cholesterol	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Cisplatin	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Confocal Laser Scanning Microscope; LSM 510/Axiovert 100M	Carl Zeiss Microscopy GmbH, Jena, Germany

Culture Tubes; Pyrex	Corning Inc., Corning, USA
DAPI	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
DMEM	PAA Laboratories, Pasching, Austria
DMEM	Lonza GmbH, Walkerville, MD, USA
DMEM: F-12	Capricorn, Ebsdorfergrund, Germany
DMEM-LG	Biochrom GmbH, Berlin, Germany
DMSO (≥ 99 %)	Acros Organics B.V.B.A., Geel, Belgium
DNA Ladder; Gene Ruler 1 kb	Fermentas Life Sciences, Vilnius, Lithuania
DOPE	Lipoid GmbH, Ludwigshafen, Germany
DPPC	Lipoid GmbH, Ludwigshafen, Germany
Dulbecco's Phosphate-Buffered Saline PBS	Biochrom AG, Berlin, Germany
EDTA	Roth, Karlsruhe, Germany
Epithelial Volt-Ohmmeter for TEER	WPI, Sarasota, FL, USA
E-Plate for xCELLigence System	Roche, Mannheim, Germany
Eppendorf Bio Photometer	Eppendorf-Netheler-Hinz, Hamburg, Germany
Ethanol	Roth, Karsruhe, Germany
Ethanol	Carl Roth GmbH + Co. KG., Karlsruhe, Germany
Ethidium Bromide	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Ethidium Bromide 1%	Roth, Karlsruhe, Germany
Extruder; Avanti Mini	Avanti Polar Lipids Inc., Alabaster, USA
Fetal Bovine Serum	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Fluorescent Mounting Medium	Dako Cytomation , Carpinteria, CA, USA
Foetal Bovine Serum	PAA Laboratories GmbH, Cölbe, Germany

Freezer (-30°C)	PORRKA, Hollola, Finland
Freezer (-86°C)	Sanyo, Bad Neudorf
Gel Doc 2000	Bio-Rad Laboratories GmbH, München, Germany
Gentamycin	Biochrom, Berlin, Germany
Glycerol	Roth, Karlsruhe, Germany
Hot Block for Tubes	HCL HBT 130, Kobe, Japan
Hydrochloride Acid	Merck, Darmstadt, Germany
Ice Machine	Ziegra, Isernhagen
Incubator Shaker	New Brunswick Scientific,USA
Isopropanol	Acros Organics, NJ, USA
Laminar Flow Hood; Labgard Class II	NuAire Inc., Plymouth, USA
Laser Scanning Microscope	Olympus, Hamburg
Leica Microsystems	Wetzler, Germany
Lipofectamine ™ 2000	Thermofischer Scientific, UK
Liquid Blocker	Daido, Sangyo, Japan
Magnetic Stirrer; MCS 66	CAT Scientific, Paso Robles, USA
Medical X-ray film	Agfa-Gevaert, Mortsel, Belgium
Methanol	Sigma-Aldrich, St. Louis, MO, USA
Micro Reagent Tubes	Sarstedt AG and Co., Nümbrecht, Germany
Microscope (Olympus A×70)	Olympus, Hamburg
Microscopy Slides	Gerhard Menzel B.V. and Co. KG., Braunschweig, Germany
MilliQ® Water	Millipore Corporation, Billerica, USA
Mini Electrophoresis System	Bio-Rad, Hercules, CA, USA
Mini Protean II Dual Slab Cell	Bio-Rad, Hercules, CA, USA
Mounting Medium; FluorSave™	Calbiochem Corporation, San Diego, USA

MTT (Thiazolyl Blue Tetrazolium Bromide)	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Nitrocellulose Membrane	Schleicher-Schuell Biosci., Dassel
Nonidet P40 Substitute NP40	Sigma-Aldrich, St. Louis, MO, USA
Olaparib	Thermofischer Scientific, UK
Optimax X-ray Film Processor	Oberstenfeld, Germany
Penicillin/ Streptomycin	Capricorn, Ebsdorfergrund, Germany
PEI MAX; Linear PEI 22 kDa	Polysciences Europe GmbH, Hirschberg, Germany
Petri Dishes; Tissue Culture grade and Suspension type	Sarstedt AG and Co., Nümbrecht, Germany
Pipetman	Gilson, Middleton, WI, USA
Pipetting Aid	Hirschmann, Eberstadt, Germany
Polycarbonate Membranes	Whatman plc, Buckinghamshire, UK
Polymin®; Branched PEI 25 kDa	BASF AG, Ludwigshafen, Germany
PowerUp SYBR Green Master Mix	Applied Biosystems, Fisher Scientific, UK
Precision Plus Protein All Blue Standards	Bio-Rad Laboratories, München
Quantstudio 5 RT qPCR instrument	Applied Biosystems, USA
Ribonuclease A	Sigma-Aldrich, St. Louis, MO, USA
RNAse free water	GE Healthcare Europe GmbH, Freiburg, Germany
Rotary Evaporator; Laborota 4000	Heidolph Instruments GmbH and Co. KG., Schwabach, Germany
Rotiphorese Gel 30	Roth, Karlsruhe, Germany
Shaking Incubator; IKA KS4000 IC	IKA Werke and Co. KG., Staufen, Germany
siRNA Dilution Buffer	GE Healthcare Europe GmbH, Freiburg, Germany
Skim milk powder	Merck, Darmstadt, Germany

Sodium Chloride	Merck, Darmstadt, Germany
Sodium Hydroxide	Fisher Scientific, Leicestershire, UK
Sodium Dodecylsulfate	Serva Electrophoresis, Heidelberg, Germany
Tetramethylethylenediamine, TEMED	Roth, Karlsruhe, Germany
Thermal Printer DPU 414	Eppendorf, NY, USA
Thermocycler	Biometra, Göttingen, Germany
Thin-Certs (0.4µmol/L)	Greiner Bio-One, Frickenhausen
Tris-HCL	Roth, Karlsruhe, Germany
Triton™ X-100	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Trypsin-EDTA	PAA, Laboratories, Pasching, Austria
Ultraviolet Transilluminator	UVP, Upland, CA, USA
xCELLigence	Roche, Mannheim
X-ray film	Agfa, Motsel, Belgium

2.1.1 siRNA sequences

(Dharmacon Inc, USA)

siRNA ID	siRNA Sequence
CRPV_E6_siRNA1	5‘-CGGAACAGGTGATACAGAT-3‘
CRPV_E6_siRNA2	5‘-CGTTGTACAGTTTGCGGAA-3‘
CRPV_E6_siRNA3	5‘-CGTTGTACAGTTTGCGGAA-3‘
CRPV_E7_siRNA1	5‘-TGTAAGCGCTGTAGGCAAATT-3‘
CRPV_E7_siRNA2	5‘-GCGACGAAGCATTAGAGAATT-3‘
CRPV_E7_siRNA3	5‘-GGAGGATCATCAAGATAGATT-3‘

2.1.2 Oligo primers

(Invitrogen, Thermofischer Scientific, Germany)

Primer ID		Primer sequence
GAPDH_1	Forward	5‘-AAGGTCATCCACGACCACTT-3‘
	Reverse	5‘-GTCTTCTGGGTGGCAGTGAT-3‘
GAPDH_2	Forward	5‘-CTGCACCACCAACTGCTTAG-3‘
	Reverse	5‘-GTCTTCTGGGTGGCAGTGAT-3‘
rRPLPO_1	Forward	5‘-GAAGCTGCTGCCTCATATCC -3‘
	Reverse	5‘-GGCCAGTAGCATATCCCTGA -3‘
rRPLPO_2	Forward	5‘-CGACGTGCAGCTGATAAAGA-3‘
	Reverse	5‘-GAGAAGGGCGAGATGTTCAG-3‘
rRPL32_1	Forward	5‘-CGGAAACCCAGAGGTATTGA -3‘
	Reverse	5‘-GCATGTGCTTGGTCTTCTTG -3‘
rRPL32_2	Forward	5‘TAAGCGTAACTGGCGGAAAC-3‘
	Reverse	5‘GCATGTGCTTGGTCTTCTTG-3‘
CRPV_E6_1	Forward	5‘-GGACTTGCCGTTTGGTTGTA-3
	Reverse	5‘-ATAGCCCCGTGCATTTGA-3‘
CRPV_E6_2	Forward	5‘GCTAGAGAAGCTGCAGCAAA-3‘
	Reverse	5‘ATAGCCCCGTGCATTTGA-3‘
CRPV_E7_1	Forward	5‘-CATTGCGACGAAGCATTAGA-3‘
	Reverse	5‘-CTTACATGGCACGGACACTG-3‘
CRPV_E7_2	Forward	5‘CAGTGTCCGTGCCATGTAAG-3‘
	Reverse	5‘ATGCGGATAGCAGTCGATTC-3‘

2.1.3 Cell lines

1. African Green Monkey SV40-transformed kidney fibroblast cell line (COS-7)
2. VX2 tumor derived cell line

2.1.4 Kits

1. First strand cDNA-Synthesis Kit (Roche Diagnostics, Mannheim)
2. QIAgen RNeasy Mini Kit (Qiagen, Hilden)

2.1.5 Antibodies

2.1.5.1 Primary antibodies

1. Anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
2. Anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
3. Anti- Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
4. Anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

2.1.5.2 Secondary antibodies

1. Anti-mouse-IgG, HRP-linked antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
2. Anti-goat-IgG, HRP-linked antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA

Experiments

Section 1

Liposomes, Polyplexes and Lipopolyplexes

As most of the physicochemical, complex stability as well as surface, morphological and structural characteristics of these liposomes, polyplexes and lipopolyplexes have been previously established [14], the main focus of this study is to elucidate the cellular uptake and cytotoxicty traits of lipopolyplexes in VX2 and COS-7 cells in which they have not been used as transfecting reagents previously.

2.2.1 Preparation of liposomes

Liposomes, polyplexes and lipopolyplexes were prepared by a previously established method [73]. Briefly, Lipids including DOPE (1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine), DPPC (1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine) and cholesterol, in a proportion of (70:15:15) respectively, were dissolved in 2 ml of 2:1 (v/v) chloroform: methanol solution in a 5 ml round bottom flask. Once dissolved, they were evaporated at 40°C on a rotary evaporator (Laborota 4000) supplied with a vacuum pump, to obtain a thin film. 10 mM HEPES buffer (pH 7.4) was used to hydrate the lipid film by sonication in a bath sonicator to obtain homogeneous suspension of liposomes. For further size reduction, these liposomes were extruded 21 times from Avanti Mini Extruder by using polycarbonate membranes (Whatman) of 400 nm and 200 nm pore size respectively. Finally, the liposomes were filtered through 0.2 µm syringe filters.

2.2.2 Preparation of polyplexes

Polyplexes were prepared with N/P ratio (ratio of PEI nitrogen atoms to nucleic acid phosphate atoms) of 9.5. lPEI was diluted in 10 mM HEPES buffer and pipetted into 1.5 ml Eppendorf tubes containing equal volume of DNA (diluted in Optima serum-free medium) or siRNA (diluted in 1x siRNA dilution buffer) and incubated at room temperature for 20-25min under a laminar airflow hood.

2.2.3 Preparation of lipopolyplexes

For preparation of lipopolyplexes, liposome to PEI mass ratio (0.39:1) was used and accordingly calculated amounts of liposomes and polyplexes were vigorously mixed in an Eppendorf tube by pipetting. Complexes were incubated at room temperature for 1 h under a

laminar airflow hood and resulting lipopolyplexes were used for transfection by mixing with cell culture medium on the cells.

2.2.4 Physicochemical characteristics

2.2.4.1 Dynamic light scattering

The size of liposomes, polyplexes and lipopolyplexes was determined using a Zetasizer Nano ZS (Malvern Instruments) instrument by dynamic light scattering. Clear disposable capillary cells (DTS1060; Malvern Instruments) were used for this purpose. For size analysis, liposomes, polyplexes and lipopolyplexes were diluted with 10 mM HEPES (pH 7.4) in a ratio of 1:100, 1:20 and 1:20 respectively. Measurements were carried out at a detection angle 173º backscatter and a wavelength of 633 nm. Laser attenuation as well as measurement position were adjusted by the instrument automatically according to the sample. Instrument worked with 15 size runs each lasting 10 s for each measurement. For data analysis, refractive index and viscosity of water at room temperature were considered as 1.33 and 0.88 mPa.s respectively. Three independent formulations were analysed and an average of their diameter was calculted.

2.2.5 Maintenance of cell culture

VX2 cells were cultivated at 37ºC and 5 % CO_2 in humid conditions in DMEM/ HAM`S F-12 medium supplemented with 10 % foetal calf serum and 100 U/ml Penicillin, 100 mg/ml Streptomycin, 50 µg/ml gentamicin, and 100 µg/ml Amphotericin. COS-7 cells [96] were cultivated at 37ºC and 5 % CO2 in humid conditions in DMEM medium supplemented with 10 % foetal bovine serum along with 100 U/ml Penicillin, 100 mg/ml Streptomycin, 50 µg/ml gentamicin and 100 µg/ml Amphotericin. Both cell lines were grown in 100 mm tissue culture dishes and passaged after reaching to 80 % confluency.

2.2.6 Cellular uptake of lipopolyplexes

For plasmid transfection, cells were grown on 6-well plates ($1x10^4$ cells per well) for 24 h until reaching to 60 % confluency. Prior to transfection, the medium on the cells was changed

to 900 µl of serum free DMEM/ HAM`S F-12 and serum free DMEM for VX2 cells and COS-7 cells respectively. VX2 and COS-7 cells were transfected with lipopolyplexes containing 2 µg plasmid DNA (E7_GFP) in a volume of 100µl Optimum serum-free media. Cells were left incubating for 4 h before adding 1ml additional media (with serum and antibiotics). One well from each plate was transfected with Lipofectamine^{TM}2000 using 2 µg control plasmid DNA (E7_GFP) according to manufacturer's protocol (Table 1), serving as a positive control for transfection experiments.

Table 1. Concentrations of Lipofectamin^{TM} 2000, dilution medium and plasmid for transfection into various types of cell culture plates

	Shared Reagents		**DNA Transfection**	
Type of Culture Plate	**Surface Area per Well (cm^2)**	**Vol.of Plating Medium (ml)**	**Vol. of Dilution Medium (µl)**	**DNA (µg)**	**Lipofectamin^{TM} 2000 (µl)**
12 well	4	1	2X100	1.6	4
6 well	10	2	2X250	4.0	10
60 mm	20	5	2X500	8.0	20

Untreated cells were used as negative control. The transfected cells were incubated for 48 h. Afterwards, cells were trypsinized and resultant cell pellets were re-suspended in 1 ml PBS (pH 7.4). 500 µl of the cell suspension was again centrifuged to obtain a cell pellet for RNA extraction (qPCR) and the remaining 500 µl cell suspension was saved for flow cytometry in FACS tubes. One ml of ice-cold ethanol was added dropwise to each FACS tube containing cell suspension, while being vortexed and stored at 4°C until FACS analysis.

2.2.7 RNA extraction and quantification

Total RNA was extracted from VX2 and COS-7 cells using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA concentration was measured on NanoDrop ND-1000 (peqLab Biotechnologie GmbH) at an extinction range of 260 nm. Also, the OD 260/230 and the OD 260/280 ratio demonstrating RNA purity were assessed. All measurements were taken as triplicates.

2.2.8 Reverse transcription

cDNA was prepared by using 500 ng of total RNA for each sample deploying the Transcriptor First strand cDNA synthesis kit (Roche, Mannheim, Germany) according to manufacturer's protocol. Briefly, 500 ng of total RNA was diluted with RNase free water to 12 µl followed by adding 1 µl of Oligo (dT) primer (500 mg/ml) in 0.5 ml RNase-free Eppendorf tubes which were then placed inside a thermocycler (TProfessional Thermocycler Biometra, Gottingen, Germany). The RT mix was heated to 65°C for 10 min, cooled on ice followed by addition of a master mix (4 µl of first-strand buffer 5X, 0.5 µl Protector RNase Inhibitor, 2 µl Deoxynucleotide mix and 0.5 µl Transcriptor Reverse Transcriptase). A final volume of 20 µl of RT mix was incubated at 50°C for 60 min followed by 85°C for 5min and 4°C hold. After cDNA preparation, all samples were diluted 2-fold with RNase free water to a final volume of 40 µl and stored at -20^{0}C until further use.

2.2.9 Primer design

For analysis of gene expression by RT qPCR, primer pairs were designed by using reference sequences of respective genes taken from GeneBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). The length of primers was set between 17 and 27 bp with an optimum length of 20 bp. The melting temperature was established between 57°C to 63°C. NCBI BLAST algorithm was used to check alignment of all primers sequences against the corresponding reference sequences [97].

Table 2. Reference sequences of CRPV oncogenes used for primer designs taken from NCBI database for RT qPCR

Gene	***Reference Sequence (NCBI ref. #)***
SE6 [Kappapapillomavirus 2]	NP_077106.1
LE6 [Kappapapillomavirus 2]	NP_077104.1
E7 [Kappapapillomavirus 2]	NP_077107.1

2.2.10 Quantitative polymerase chain reaction (qPCR) assay

Real time quantitative PCR analysis was performed by using PowerUp™ Sybr Green Master Mix (Applied Biosystems, Darmstadt) according to manufacturer's protocol. Briefly, both 5′ and 3′ primers were diluted with PCR grade water to a final conc. of 10 µmol/L and 1 µl of each primer was added to 6 µl PCR mix followed by addition of 2 µl of cDNA. 10 µl of this mix was put in each well of 96-well optical plate and wells were sealed with ABI MicroAmp™ Optical Adhesive Film and centrifuged briefly to collect the contents and ensure that no bubbles inhibit the signal detection. All samples were prepared in triplicates with 5% excess for pipetting loss. QuantStudio™ 5 system (Thermo Fisher Scientific) was used for quantitative PCR. The following thermal profile was applied: 1 cycle at 50°C for 2 min, at 95°C for 2 min, 40 cycles at 95°C for 3 s and 60°C for 30 s followed by 4°C hold. Gene expression was evaluated in term of fold induction respective to the untreated cellular population (control) by $2^{-\Delta\Delta ct}$ method [98].

Where

ct = Number of amplification cycles required to reach a fixed signal threshold

Δct = Normalized expression values for each condition (untreated or treated)

ΔΔct = Direction and the size of the change in gene expression between two conditions.

The delta-delta-ct value is the logarithm of the ratio of concentrations, also kown as "log fold-change".

Assessment of results was carried out as follows: genes were considered differently expressed when their change was greater than ± 2.5 folds with respect to the transcript levels of the untreated sample [16]. All experiments were conducted in triplicates.

2.2.11 *In vitro* cytotoxicity assay

To estimate the extent of cellular cytotoxicity as a result of using lipopolyplexes, VX2 and COS-7 cells were seeded in 96-well plates at a density of $2x10^3$ and $4x10^3$ cells per well respectively and transfected with 0.25 µg of plasmid DNA (E7_GFP) by using lipopolyplexes and Lipofectamine™2000 using the same protocol as described previously. 48 h after transfection, plates were washed twice with PBS containing Ca^{++} and Mg^{++} followed by addition of 200 µl (2 mg/ml) MTT dye per well and plates were incubated for 4 h to allow formazan formation. 1 % Triton™X-100 was deployed as a positive control.

Untreated cells were used as negative control and wells containing only medium were taken as background control. Once formazan crystals were formed depending on the presence of viable cells and MTT interaction, medium was removed from the wells and replaced with 200 µl of DMSO. Plates were incubated on a shaker (KS4000IC, IKA Werke, Staufen, Germany) for 30 min at 120 rpm. The absorbance of samples was measured at 570 nm in a FLUOStar^{TM} Optima plate reader [99].

2.2.12 FACS analysis in E7_GFP expressing cells

VX2 and COS-7 cells were transfected with E7_GFP plasmid DNA using lipopolyplexes as transfecting reagents and also by Lipofectamine^{TM}2000 used as positive control for comparison. After 48 h, cells were trypsinized, fixed in 70 % ethanol and stored at 4^0C. Prior to FACS analysis, cells were centrifuged at 300 g for 10 min, ethanol was removed and the cell pellet washed twice in PBS. The pellet was dissolved in 500 µl PBS in a FACS tube by gently vortexing followed by flow cytometry analysis on (BD LSR II Becton Dickinson, Franklin Lakes, New Jersey). Dead cells (debris) were excluded from analysis by deploying forward (FSC) and side scatter (SSC) parameters. GFP expression was detected by measurement of the fluorescence (argon laser at 530/30 nm fluorescence with a bandpass filter at FL1 channel).

2.2.13 Fluorescence microscopy

COS-7 cells were seeded with a density of $5x10^3$ cells/well and $1x10^4$ cells/well respectively, onto sterilized cover slips (24 mm diameter) in 6-well plates and incubated for 24 h prior to transfection. Cells were transfected with E7_GFP plasmid DNA as described earlier. After 48 h, media was removed and cells were washed with cold PBS containing Ca^{++} and Mg^{++} (pH 7.4) and fixed with 100 % methanol for 10 min at -20°C followed by washing the cells in PBS. Lastly, coverslips were mounted on the cells and sealed with transparent nail polish. Confocal laser scanning microscopy (Zeiss Axiovert 100 M, Carl Zeiss Microscopy GmbH, Jena, Germany) was deployed to examine the cells with band pass filters of 505–530 nm for GFP detection.

Section II

siRNA-mediated knockdown in VX2 cells

2.3.1 Detection of CRPV E6 and CRPV E7 in VX2 cells

VX2 cells were grown in DMEM/ Ham`s F-12 medium supplemented with 10 % foetal calf serum and antibiotics. After 48 h, cells became 80-90 % confluent and harvested to obtain a cell pellet for RNA extraction. RNA was prepared by following the protocol mentioned in section (2.2.7). RNA concentration was measured with the Nano Drop ND-1000 system (peqLab Biotechnologie GmbH) and 1 µg RNA was used to make cDNA following the protocol mentioned above (2.2.8). qPCR was done by using especially designed primers for rabbit GAPDH, CRPV E6 and CRPV E7. cDNA prepared from RNA derived from rabbit skin was used as a negative control.

2.3.2 Titration of siRNA concentrations for the knockdown of E6 and E7

To determine appropriate concentration of siRNA for the knockdown of CRPV E6 and E7 oncogenes in VX2 and COS-7 cells, a range of siRNA concentrations (12.5-150 nmol/L) was assessed and following concentrations of siRNA were selected for further studies:

Table 3. siRNA concentrations used for knockdown of CRPV E6 and E7 in VX2 and COS-7 cell lines using lipopolyplexes as transfecting reagent

siRNA Specificity	***Cell Line***	***Concentration of siRNA used for gene knockdown***	***Incubation Time***	***Transfection Reagent***
NT (Non target)	VX2	50 nmol/L	48 hours	lipopolyplexes
E6	VX2	50 nmol/L	48 hours	lipopolyplexes
E7	VX2	50 nmol/L	48 hours	lipopolyplexes
E6+E7	VX2	25 nmol/L+25 nmol/L	48 hours	lipopolyplexes
NT (Non target)	COS-7	150 nmol/L	48 hours	lipopolyplexes
E6	COS-7	150 nmol/L	48 hours	lipopolyplexes
E7	COS-7	100 nmol/L	48 hours	lipopolyplexes

2.3.3 Transfection of siRNA loaded lipopolyplexes in VX2 cells

VX2 cells were directly transfected with siRNA containing lipopolyplexes as VX2 cells endogenously express E6 and E7 oncogenes. siRNAs for knockdown of CRPV E6 and E7 oncogenes were used as described in Table 3. After 48 h incubation, cells were trypsinized and pellets were saved for qPCR and Western Blot analysis at -80°C. For flow cytometry, VX2 cells were fixed in 70 % ethanol as described above in (2.2.6).

2.3.3.1 RT qPCR analysis

After siRNA knockdown, cells were analysed by RT qPCR method as described above in (2.2.10).

2.3.3.2 Western blot analysis

Western blot analysis was performed according to the protocol described in (2.4.1.2.2) except for using a different primary antibody (mouse monoclonal anti-PCNA sc-56, clone PC-10, 1:50, Santa Cruz Biotechnology)

2.3.3.3 MTT assay

VX2 cells were seeded in 96 well plates at a density of $2x10^3$ cells per well. After 24 h, the medium was removed and replaced with 90 µl of serum free medium along with 10 µl of siRNA loaded lipopolyplexes. 8 wells were treated with Non-Target siRNA loaded lipopolyplexes, 8 wells with CRPV_E6 siRNA loaded lipopolyplexes and 8 wells with CRPV_E7 siRNA loaded lipopolyplexes. All of the above-mentioned siRNAs were used at a concentration of 50 nmol/L. After 48 h, the MTT assay was performed as described above.

2.3.3.4 Phenotype specificity analysis

As observed during initial studies, VX2 cells exhibit two phenotypes in cell culture which grow side by side. One phenotype was called large VX2 cells population and the other Phenotype was called small VX2 cells population. In order to see if the knockdown of CRPV_E6 or CRPV_E7 or both genes simultaneously, affects the phenotype of these cells,

VX2 cells were seeded on a 6-well plate at a density of $2x10^3$ cells per well. After 24 h incubation, when cells reached 60-70 % confluency, medium was removed and replaced with 800 µl of serum free DMEM/ HAM'S F-12 medium. One of 6 wells was treated with 200 µl of Non-Target small RNA (50 nmol/L) loaded lipopolyplexes, one well with CRPV_E6 siRNA (50 nmol/L) loaded lipopolyplexes, one with CRPV_E7 siRNA (50 nmol/L) loaded lipopolyplexes and one well with both CRPV_E6 siRNA and CRPV_E7 siRNA (25 nmol/L+25 nmol/L) loaded lipopolyplexes. After 4 h incubation, 1 ml DMEM/ HAM`S F-12 medium with serum and antibiotics was added to each well. After 48 h incubation, VX2 cells treated with siRNA loaded lipopolylexes were observed under the microscope and images were taken of each well. The phenotypes in each well were quantified with the WIMasis image analysis software (Onimagin Technologies SCA, Cordoba, Spain) on the basis of branching point count evaluation [100].

2.3.3.5 Cell cycle analysis by FACS

Cell cycle phase distribution was analysed after Propidium Iodide (PI) staining using flow cytometry. 48 h post transfection and knockdown of CRPV E6 and E7 genes, cells were harvested followed by washing cells in cold PBS with subsequent fixation 70 % ethanol. For flow cytometry assay, cells were washed twice in PBS, resuspended in PBS containing 50 µg/ml RNase A (Sigma Aldrich) and incubated for 30 min at 37°C. Afterwards, 50 µl of Propidium Iodide stock solution (1 mg/ml) was added into cells suspension (final concentration: 25 µg/µl) to stain the genomic DNA and samples were passed through (BD FACS Calibur Flow Cytometer (Becton Dickinson, Heidelberg, Germany). Maximum excitation range of fluorescence used for detection of PI bound to DNA was adjusted to 536 nm, and emission was set to 617 nm whereas 488 nm was optimal for excitation of PI. Three independent experiments were carried out for cell cycle analysis of VX2 cells analysing the effect of E6 and E7 SiRNA knockdown. Cell cycle stages were calculated by using the ModFit LT software for Mac systems.

2.3.3.6 Real time cellular analysis

Cell adhesion to adjacent cells and to basal extracellular matrix (ECM) is the primary requirement for the existence and integrity of all multicellular organisms. Trans-membrane heterodimeric family of receptors called integrins are responsible for ECM adhesion of cells

by controlling numerous cytoskeletal proteins attached to integrin tails, a vast array of signaling and scaffolding. Together, these adhesion components recruit a highly complex and dynamic system which regulates various cellular mechanisms and functions including cell proliferation, survival, differentiation and migration. Disruption in integrin-mediated cell adhesion may be the result of various pathological conditions including cardiovascular diseases, bleeding disorders and cancer [101-104]. xCELLigence RTCA system monitors changes in cell-ECM adhesion quantitatively.

Impedance measurement depends on the extent of cell confluency and is expressed as Cell Index (CI). The cell layer growing on and between gold microelectrodes causes disturbance in the current flow thus enhancing electrode impedance (resistance to alternating flow of current). The magnitude of Cell Index depends upon the number of cells, their size and the degree of firmness of cell adhesion to the substrate coating the plates [105].
Cell Index = Rn – Rb / Rb
Rn = electrode/cells Impedance in a well
Rb = background Impedance in a well with medium alone.

Migration, proliferation and cell-ECM adhesion in untreated and siRNA treated VX2 cells were measured with the xCELLigence system (Roche, Mannheim, Germany). E-plate (specifically designed for Impedance measurement of cells, Roche, Mannheim, Germany) was prepared by adding 150 µl of DMEM/ Ham's F-12 medium containing serum and antibiotics into the wells. Equilibration was achieved by placing the plate into the xCELLigence station and base-line electrical resistance was gauged to make certain that all connections to the wells were working in appropriate limits. E-plate was taken out and 50 µl of VX2 cells suspension was added to each well with a cell density of $4.5x10^3$ cells per well and then placed back in xCELLigence station at 37°C for 24 h.

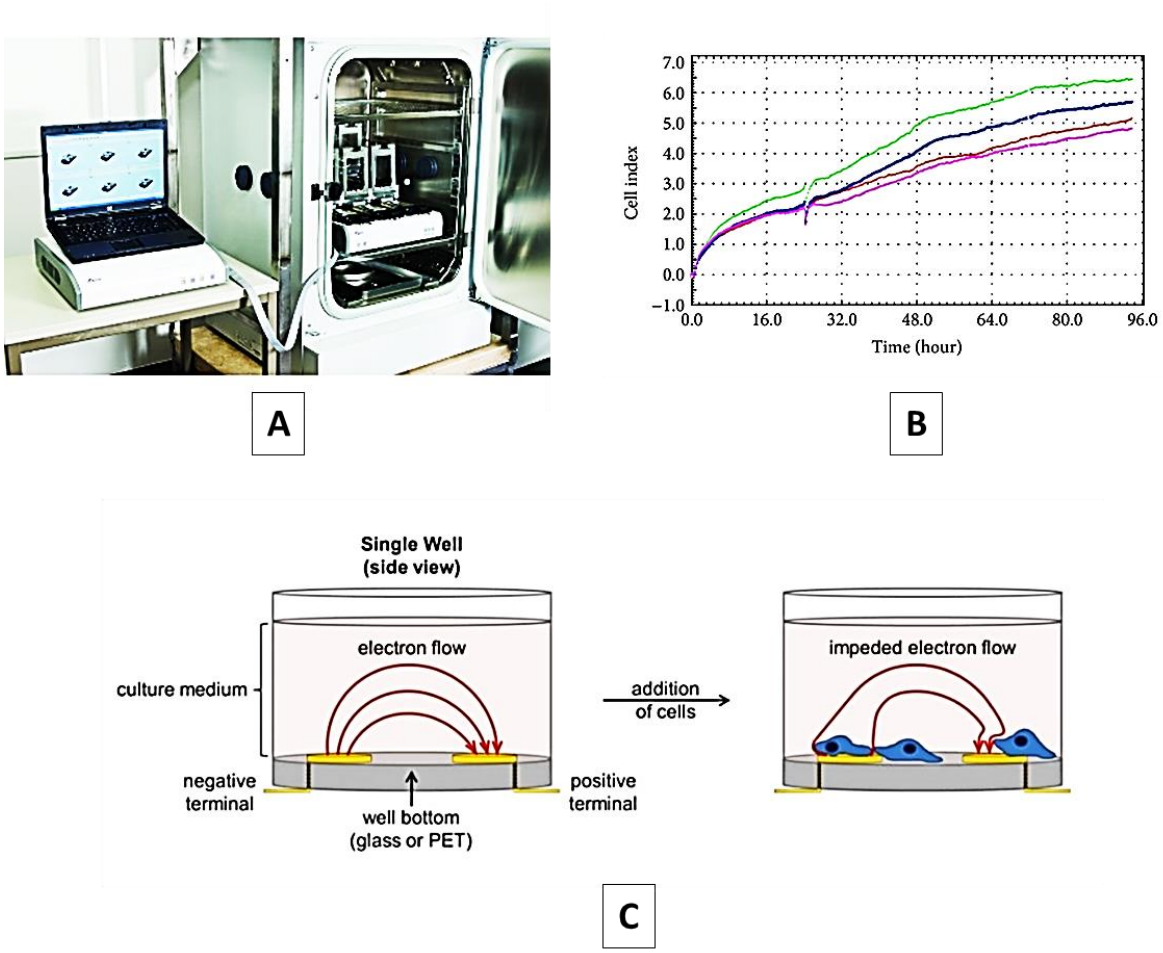

Figure 3. ***xCELLigence system (A)*** *The system consists of: real-time cell analysis (RTCA) Analyser, RTCA SP Station, RTCA Control Unit and single use E-Plate 96.* ***(B)*** *Cell Index values, derived from the measured impedances, are continuously displayed with the Software.* ***(C)*** *Cells attaching on the top of the electrode increase the impedance. (Modified from www.roch-applied-science.com/sis/xcelligence/index)*

Cell Index was monitored and once VX2 cells started to adhere to the base, E-plate was taken out again and cell culture medium was removed and replaced by 175 µl fresh medium and 25 µl of siRNA (E6 150 nmol/L, E7 150 nmol/L and E6+E6 75+75 nmol/L) loaded lipopolyplex solution. The E-plate was placed again in the incubation chamber of xCELLigence instrument for continuous real time monitoring of cell proliferation (every 15 min for 72 hours).

2.3.3.7 Wound healing assay

VX2 cells were seeded at a density of $1x10^4$ cells per well in a 6-well plate in DMEM/ Ham's F-12 medium containing serum and antibiotics. After 24 h, the medium was replaced with 900 µl of serum free DMEM/ HAM'S F-12 medium followed by addition of 100 µl siRNA loaded lipopolyplexes solution in each well. Non-Target siRNA 50 nmol/L, E6 siRNA 50 nmol/L, E7 siRNA 50 nmol/L, and E6 siRNA+E7siRNA 25+25 nmol/L).
After 4 h, 1 ml DMEM/ HAM'S F-12 medium was added 24 h post siRNA transfection, the layer of cells was scratched by using a 100 µl sterile pipette tip. Cells were washed with PBS to remove dislodged cells; medium was added again and cells were left in incubator under standard conditions. After 24 h at three different positions, micrographs were taken to be analysed with the WIMasis image analysis software (Onimagin Technologies SCA, Cordoba, Spain) and Fiji ImageJ software. The rate of migration was defined as the percentage of wound closure area [106].

2.3.4 Knockdown of CRPV E6 and E7 in VX2 tumor suspension cells

In this experiment VX2 tumor tissue was taken out from tumor bearing cottontail rabbit and triturated to a cell suspension. Out of this raw tumor suspension, 10 µl was taken and put in a 50 ml sterilized tube containing 36 ml DMEM: F12 medium with 10 % FCS serum and antibiotics. This diluted VX2 tumor suspension was passed through a 0.5 mm mesh. 2 ml of this cell suspension was added to each well of three 6-well plates. This cell suspension was directly subjected to transfection by different concentrations of siRNA targeting CRPV E6 and E7 using lipopolyplexes as transfecting reagents as described above.

Section III

Exogenous knockdown of CRPV E6 and E7 by siRNA loaded lipopolyplexes

2.4.1 Validation of siRNA localization on the target transcripts transfected into a non-CRPV cell line

Target site accessibility is crucial for successful siRNA knockdown. To confirm that siRNAs against CRPV E6 and E7 oncogenes have no off-target effects and are suitable for the knockdown of these genes, a series of experiments was contrived in which a non-CRPV cell line, COS-7 was used.

2.4.1.1 Transient transfection of cloned plasmids in COS-7 cell line

COS-7 cells were seeded on 6-well plates at a density of $1x10^4$ cells per well in DMEM medium with 10 % FCS and antibiotics. After 24 h, COS-7 cells became 60-70 % confluent and were transfected with 2 µg of CRPV_E6_GFP and CRPV_E7_GFP expressing plasmids, separately, using lipopolyplexes as transfecting reagents. After 24 h incubation, fluorescence microscopy was performed to evaluate transfection efficiency (confocal laser scanning microscope band pass filters of 505–530 nm, Zeiss Axiovert 100 M, Carl Zeiss Microscopy GmbH, Jena, Germany).

2.4.1.2 siRNA knockdown of CRPV E6 and CRPV E7 in COS-7 cells

After validation of E6 and E7_GFP gene expression by fluorescence microscopy after transfection, COS-7 cells were transfected with CRPV_E6 or CRPV_E7 specific siRNA using siRNA loaded lipopolyplexes as described earlier. Non-target siRNA was used as a control to account for unspecific effects of siRNA transfection. The moderate concentration of siRNA for the knockdown of CRPV_E6 and CRPV_E7 was achieved after titration through multiple experiments, as the expression of CRPV_E6 and CRPV_E7 in transiently transfected COS-7 cells is much higher than in VX2 cells in which these genes are a part of the genome. So, the concentration of siRNA used for the knockdown of CRPV_E6 and CRPV_E7 in VX2 cells, was not comparable or relative to the concentration of siRNA, used for the knockdown of the same genes in transiently transfected COS-7 cells. In COS-7 cells, 150 nmol/L siRNA conc was used for CRPV E6 and 100 nmol/L siRNA for CRPV E7 SiRNA knockdown. Non-Target siRNA was used as a control in the same concentration as used for E6/E7 specific knockdown. Subsequently, cells were trypsinized and pellets were saved for RT qPCR and Western Blot assays respectively. For flow cytometry analysis, COS-

7 cells were fixed in 70 % ethanol as described previously.

2.4.1.2.1 qPCR analysis

RT-qPCR analysis was performed using the same protocol as described previously. GAPDH was chosen as house-keeping gene for normalization.

2.4.1.2.2 Western blot analysis

24 h after siRNA transfection, cell pellets were exposed to lysis buffer containing 2 mM EDTA, 1 % NP40, 137 mM NaCl, 20 mM TRIS/HCl (pH 8.0), 10 % glycerol along with protease and phosphatase inhibitors (Sigma Aldrich). Lysates were mixed using an orbital shaker for 1 h at 4 °C and then centrifuged at $12x10^3$ g for 20 min at 4 °C. Supernatant containing the whole cellular protein was transferred to a clean tube and protein concentration was measured with the Bradford method using a spectrophotometer (Bio-Rad Labs, Munchen, Germany). A 12 % sodium dodecyl sulfate acrylamide gel (SDS-PAGE) was used for protein separation according to their size. Each lane was loaded with 35 µg whole cells lysate. For size comparison, Precision Plus Protein™ Standard (161-0373) from Biorad (Hercules, CA, USA) was used as control. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes which were blocked with 3 % milk/PBS for 1 h at room temperature and incubated with the primary antibody (mouse monoclonal Anti-GFP, Santa Cruz, CA, USA) overnight at 4 °C. Next day, membranes were washed thrice in 3 % milk/PBS for 15 min and incubated with an HRP-coupled secondary antibody (mouse-IgGk BP-HRP, 1:2000, Santa Cruz, CA, USA) at room temperature for 1 h. Membranes were washed thrice again to remove excessive antibody and bands were visualized with the enhanced chemiluminescence (ECL) method (Amersham Biosciences, Buckinghamshire, UK) on X-ray film (Agfa, Cologne, Germany).

2.4.1.2.3 Flow cytometry analysis

siRNA knockdown of CPRV E6 and E7 in COS-7 cells should also cause a decline in the fluorescence expression of GFP which was tagged along with the respective gene. To confirm this, GFP expression in COS-7 cells transfected with CRPV_E6 and CRPV_E7 siRNA loaded lipopolyplexes of was analysed by flow cytometry.

COS-7 cells were fixed in 70 % ethanol as described earlier. Fixed cells were then centrifuged at 300 g for 10 min, ethanol was removed, the pellet was washed twice in PBS and resulting pellet was dissolved in 500 µl PBS followed by flow cytometry analysis (BD LSR II, Becton Dickinson, Franklin Lakes, New Jersey). Dead cells were excluded from analysis using forward and side scatter parameters. GFP expression signal was detected by exciting an argon laser at fluorescence intensity 530/30 nm.

2.4.1.2.4 Fluorescence microscopy

Change of GFP expression in COS-7 cells after siRNA knockdown with CRPV E6 and E7 siRNA was evaluated by (Confocal laser scanning microscope, Zeiss Axiovert 100 M, Carl Zeiss Microscopy GmbH, Jena, Germany)

Section IV

Limiting factors affecting gene therapy of CRPV carcinoma

2.5.1 Course of CRPV E6 and E7 oncogene expressions under cellular stress

Cancer cells exposed to serum starvation *ex vivo* partially mimic metabolically distressed cells trying to adapt to a changed environment *in vivo* by inducing exaggerated gene expression and signal transduction so that the tumor can grow continuously and least perturbed. One study shows that the altered protein and phosphoprotein levels caused by serum starvation can reflect the adapted tumor phenotype which can provide some targets for cancer therapy [107]. Oncogenes may change their gene expression levels in case of stress induction. This study is aimed to assess changes in transcript levels of CRPV E6 and E7 oncogenes in VX2 cells in the presence of different stress induction scenarios. This may give us an insight about the importance of E6 and E7 oncogenes for the survival of cancer cells in harsh environments and how targeting these oncogenes can contribute to CRPV cancer therapy.

2.5.1.1 Serum Starvation

For serum starvation experiments, VX2 cells were grown in DMEM/ HAM`S F-12 medium, without addition of serum or antibiotics. Different experimental designs of serum starvation were applied to witness the change in expression of CRPV E6 and E7 in VX2 cells as enlisted below:

1) VX2 cells were grown in serum free DMEM/ HAM`S F-12 medium. After three days, cells were trypsinized and passaged again in serum free DMEM/ HAM`S F-12 medium. Following three days of incubation, cells were harvested and cell pellet was obtained for RNA extraction, cDNA preparation and RT qPCR analysis.
2) VX2 cells were cultured in serum free DMEM/ HAM`S F-12 medium for 24h. Afterwards, cells were harvested, passaged again in serum free DMEM/ HAM`S F-12 medium and left to grow for 24 h followed by passaging again in serum free DMEM/ HAM'S F-12 medium. This cycle was repeated for seven days. Each day, after trypsinization of VX2 cells, half of the cell suspension was centrifuged and resultant cell pellet was stored for RT qPCR analysis, while half of the cell suspension was passaged again to serum free DMEM/ HAM'S F-12 medium and incubated for 24 h. VX2 cells in normal medium containing serum and antibiotics were passaged in the same way for seven days.

3) VX2 cells were cultured in serum free DMEM/ HAM`S F-12 medium in four (10 mm) petri dishes and incubated for 24 h. Following scenarios of serum starvation were applied in this case:

   a) VX2 cells were harvested from dish number 1 after 24 h and the cell pellet was stored for RNA extraction and subsequent RT qPCR analysis.
   b) VX2 cells were harvested from dish number two after 48 h and the cell pellet was stored for RNA extraction and subsequent RT qPCR analysis.
   c) VX2 cells were harvested from dish number three after 1 week without any medium change, and the cell pellet was stored for RNA extraction and subsequent RT qPCR analysis.
   d) VX2 cells were harvested from dish number four after two weeks without any medium change, and the cell pellet was stored for RNA extraction and subsequent RT qPCR analysis

4) VX2 cells were cultured in serum free DMEM/ HAM`S F-12 medium and incubated for one week without trypsinization. Medium was changed during this period. After seven days incubation in fresh serum free medium daily, cells were harvested for RNA extraction, cDNA preparation and RT qPCR analysis.

In all of the above conditions applied, after every trypsinization, $1x10^4$ VX2 cells were seeded to pass to the next level of serum starvation. No media with serum was used in between any of the above-mentioned serum-starved conditions. At the end of every experiment of serum starvation, VX2 cells pellet was collected, RNA was extracted, cDNA was prepared and RT qPCR analysis was performed.

2.5.1.2 Exposure to different concentrations of Cisplatin

VX2 cells were seeded in DMEM/ HAM`S F-12 media with 10 % FCS and antibiotics, in the usual fashion mentioned previously. After 24 h, when cells reached 50-60 % confluency, cells were exposed to different concentrations of cisplatin (0.01, 0.1, 1, 10, 50 and 100 µmol/L). After 24 h cisplatin exposure, VX2 cells were trypsinized and cell pellets were collected for RNA extraction, cDNA preparation and RT qPCR analysis by following the protocols described previously. Expression of CRPV oncogenes (either CRPV E6 or CRPV

E7 or both) was analysed.
cDNA prepared from normal VX2 cells grown in DMEM/ HAM`S F-12 medium with serum and antibiotics was used as a positive control in all RT qPCR experiments against cisplatin treated VX2 cells.

2.5.1.3 Effect of 4 Gy radiation and 40 µmol/L Olaparib on VX2 Cells

Olaparib is a PARP, poly (ADP-ribose) polymerase, inhibitor which limits DNA repair as a result of oxidative stress and is used to sensitize cancer cells to proton radiation [108]. Previous studies have shown that Olaparib causes papilloma virus positive HNSCC cell lines to become more sensitive for radiation thus increasing vulnerability of these cell lines for radiotherapy [109-111]. VX2 cells were exposed to Olaparib (40 µmol/L), which was non-cytotoxic and increased sensitivity of VX2 cells towards radiation, followed by radiation (4 Gy) to observe the change in expression of CRPV E6.

For this study, VX2 cells were grown in DMEM/ HAM`S F-12 medium with serum and antibiotics for 24 h. When cells reached 50-60 % confluency, Olaparib (dissolved in DMSO, final conc. 40 µmol/L) was added to the medium above cells and incubated for further 24 h. Cells were exposed to ionized radiation (4 Gy) using Gamma cell 1000 Elite irradiator (Theratronics, Ottawa, Canada) with a ^{137}Cs source. Afterwards, VX2 cells were trypsinized, cell pellet was obtained for RNA extraction, cDNA preparation and subsequent RT qPCR analysis to check the expression of CRPV E6 oncogene in irradiated VX2 cells compared to untreated VX2 cells.

Statistical Analysis

All experiments were carried out in triplicates and results were shown as mean ± standard deviation. Statistical significance differences were analysed by two-tailed Student's t-test. Results were considered significant with a probability $p < 0.05$. Statistical differences were indicated as "*" $p < 0.05$, "**" $p < 0.01$, "***" $p < 0.001$. Graphical representation of results was provided by using GraphPad Prism version 5 for Windows, GraphPad Software (La Jolla California, USA)

Part III: Results and discussion

Section 1

Liposomes, Polyplexes and Lipopolyplexes

3.1 Physicochemical properties

The basic prerequisite for efficient genetic material delivery into the cells is micro-sized, marginally cationic and physiologically intact complexes. Hydrodynamic diameter of these complexes is primarily their size which is measured in a hydrated, non-spherical state by using dynamic light scattering. The measurements for size determination by laser Doppler velocimetry was carried out at a neutral pH (7.4) by using 150 mM NaCl in 20 mM HEPES buffer as dilution medium in order to mimic physiological *in vivo* conditions [112].

3.1.1 Hydrodynamic diameter

Liposomes and complexes were evaluated for hydrodynamic diameter measurement by using size by intensity function of dynamic light scattering. In affirmation with former research studies, polyplexes exhibited larger hydrodynamic diameter if their N/P ratio was increased for both linear and branched PEI because at higher N/P ratio, polyplexes tend to form aggregates shortly after complexation [113]. For N/P ratio of 3 to 20, bPEI complex size was decreased from 295.9 ± 6.3 nm to 189.5 ± 6.1 nm respectively. In case of lPEI, for N/P ratio of 2.5, the size of complex was 208.5 ± 5.5 nm whereas for N/P ratio 22, it was 125.7 ± 8.5 nm. These size differences however, showed no prominant changes in the diameter of pDNA or siRNA containing polyplexes which was in accordance with previous studies [114].

In this study, formulation containing DOPE, DPPC and cholesterol was analysed. Liposomal formulation was extruded through a mini extruder in order to achieve homogenous unilamellar consistency with reduced size [115]. In order to correlate the size of liposomes with the size range of polyplexes (150-180 nm), the liposomal formulation was passed through 400 and 200 nm polycarbonate membranes through extrusion process. Hence, a monodisperse formulation of liposomes with an average size range of 160-180 nm was obtained having PDI (polydispersity index) of 0.1.

As higher concentration of DOPE in the formulation makes liposomes more vulnerable to aggregate under physiological conditions and hexagonal structure of DOPE is restored after some time, a cautious amount of 70 % of DOPE was used which kept its fusogenic properties stable [116]. A detailed account of N/P ratios of polyplexes and mass ratios of polyplexes to liposomes gave a conclusive idea for requirement of the desired size range of lipopolyplexes. As previous studies indicate that for efficient endocytosis and cellular uptake, complexes should be of 200 nm or less in size [117], so liposomes were prepared with a size range of 170-210 nm.

3.1.2 Zeta Potential

Zeta potential is described as the electro-kinetic potential in colloidal dispersions. It depends upon the electrostatic potential surrounding the double layer of a nanoparticle. The ability of nanoparticles such as liposomes to permeate biological layers mainly depend upon zeta potential. Therefore, zeta potential is a very important factor which affects cellular interaction and uptake of complexes. Two of the most essential factors affecting on zeta potential are pH and ionic strength. The gene delivery vehicles need to have a weak positive charge for an efficient transfection of genetic material into the cells [118, 119]. Because of the presence of DOPE, liposomal formulation showed either neutral or negative zeta potential.

For polyplexes preparation, if N/P ratios of both linear and branched PEI were increased, zeta potential was also found to be increased, however, in case of branched PEI, zeta potential elevated drastically whereas for linear variant of PEI, zeta potential increased only slightly. As high cationic charge of PEI is considered responsible for higher cytotoxicity, only N/P ratios with a cationic charge less than +30 mV were used [120].

Various factors effect on the complexation of liposomes with nucleic acids, nanoparticles or complexes such as physical interactions, electrostatic combinations, structural rearrangements either individually or in combination [121]. Liposomes play a vital part in physicochemical characteristics of a formulation along with polyplexes [122]. Liposomes showed a highly negative zeta potential which helped a great deal in neutralizing the high cationic charge of PEI thus making lipopolyplexes less cytotoxic.

Table 4: Physicochemical properties of liposomes, polyplexes and lipopolyplexes. Size and zeta potential are expressed as a mean ± standard deviation of three independent measurements (n=3)

Formulation	Size (nm) ± SD	Zeta Potential (mV) ± SD	PDI
Liposomes (mol %)			
DOPE:DPPC:Cholesterol (70:15:15)	174.7 ± 3.3	-30.9 ± 2.0	0.09
Polyplexes			
Branched PEI (N/P 15)	180.5 ± 11.6	+ 27.3 ± 4.4	0.35
Linear PEI (N/P 9.5)	166.2 ± 8.7	+ 19.1 ± 2.7	0.24
Lipopolyplexes with lPEI polyplexes			
DOPE:DPPC:Cholesterol (70:15:15)	201.1 ± 5.9	+ 3.7 ± 2.1	0.19
Lipopolyplexes with bPEI polyplexes			
DOPE:DPPC:Cholesterol (70:15:15)	194.0 ± 17.3	+ 9.5 ± 3.7	0.38

Size and zeta potential are expressed as a mean ± standard deviation of three independent measurements (n=3). As linear PEI polyplexes with N/P ratio 9.5 showed better trasfection abilities and found more stable than branched PEI polyplexes with N/P ratio 15 [14], linear PEI containing lipopolyplexes were selected for further studies.

3.2 Evaluation of transfection efficiency of lipopolyplexes

3.2.1 Flow cytometry analysis

Transfection of nucleic acids such as plasmid DNA or RNA is achieved by various methods involving physical, chemical or biological methods or a combination of these [123]. Various factors influence transfection efficiency, cellular toxicity and the extent of gene expression. To measure the impact of these factors on the efficiency of a transfection method or reagent, a reliable detection system is required [124]. An emerging trend among sensitivity-based assays is the use of reporter proteins for example green fluorescent protein (GFP) or firefly Renilla luciferase [125]. Although Luciferase assays are widely used for its sensitivity and suitability to determine relative transfection efficiencies of different samples, major limitation of this method is the need to lyse the cells therefore no quantification of cellular toxicity is possible. GFP reporter assays require the cellular uptake to be visualized by fluorescence microscopy which is laborious and subjective [126].

However, flow cytometry presents a suitable alternative to analyse GFP transfection efficiency allowing for quantification of GFP expression in single cells [127]. To measure GFP expression by flow cytometry, GFP alone or GFP-fused proteins or co-transfection of GFP with candidate gene expressing plasmid is done. All these techniques have their drawback such as competitiveness in expression between two plasmids [128].

In this study plasmid was tagged with GFP and its expression was directly analysed by flow cytometry after transfection. Expression of CRPV E7_GFP plasmid was measured in two cell lines, VX2 and COS-7, by using lipopolyplexes as transfecting reagents. GFP expression in both cell lines was compared with non-transfected cells (negative control) and cells transfected with Lipofectamine 2000^{TM} (positive control).

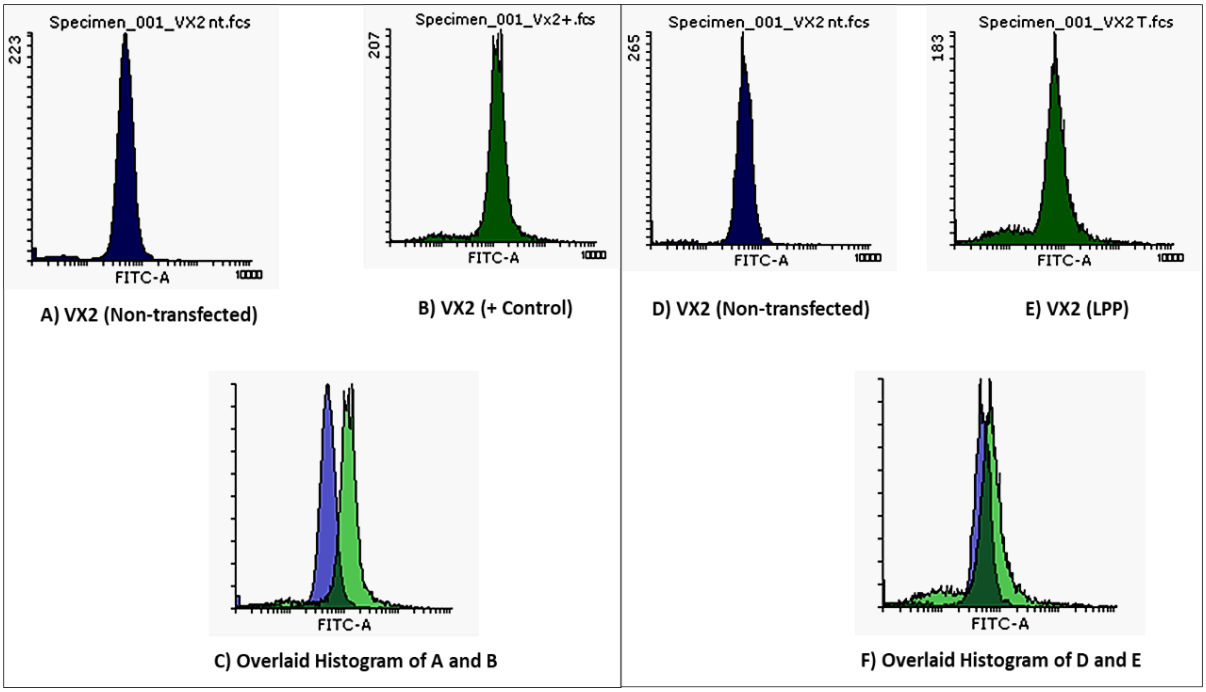

Figure 4. *Flow cytometry analysis of VX2 cells transfected with E7_GFP plasmid A). Non-transfected B) Transfected with Lipofectamine 2000™ C) Overlaid histogram, D) Non-transfected E) transfected with lipopolyplexes, F) Overlaid histogram*

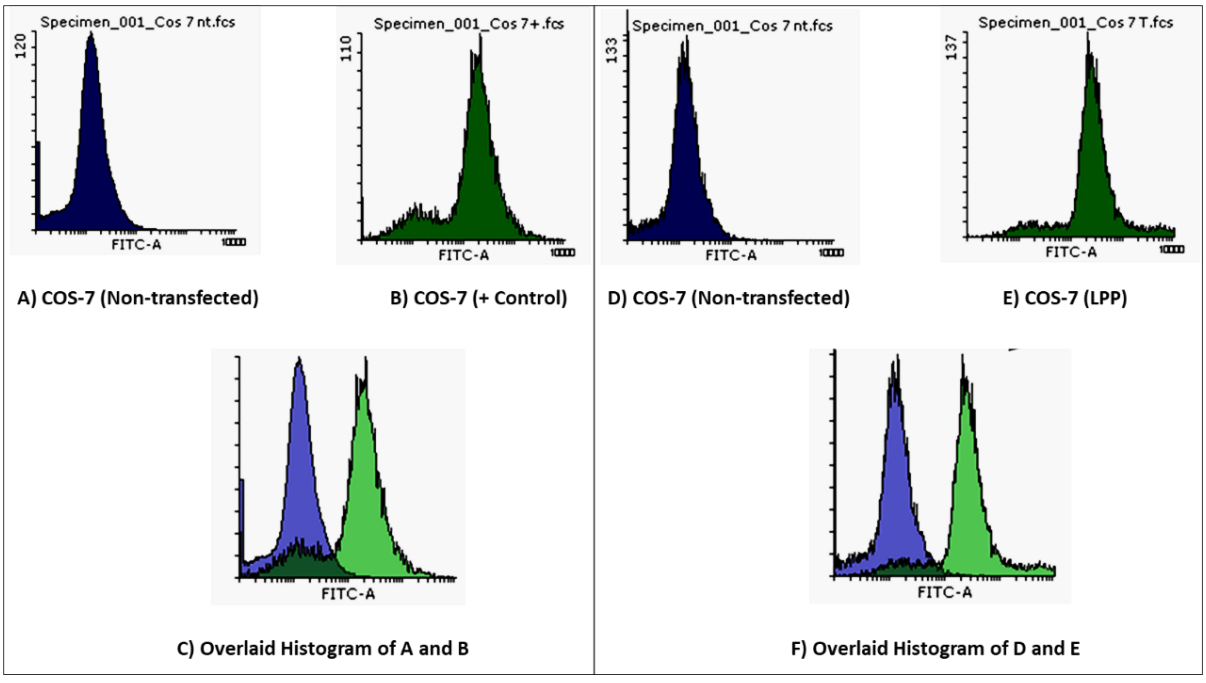

Figure 5. *Flow cytometry analysis of COS-7 cells transfected with E7_GFP plasmid A). Non-transfected B) Transfected with Lipofectamine 2000™ C) Overlaid histogram, D) Non-transfected E) transfected with lipopolyplexes, F) Overlaid histogram*

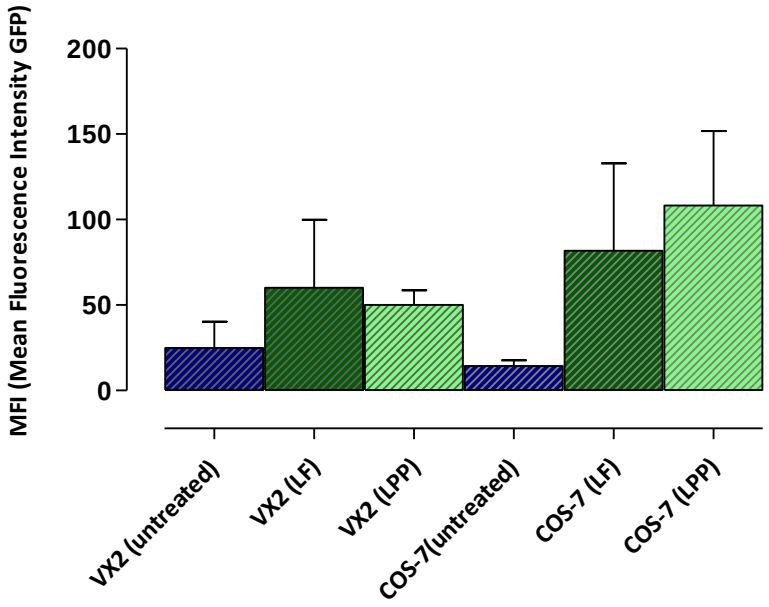

Figure 6. *Quantitative analysis of GFP expression in COS-7 and VX2 cells*

Fluorescnece intensity of GFP was calculated as geometric mean by the formula given below:

$$\text{G-mean}=10^{\Sigma \log X(i)/n}$$

where $X(i)$ = channel or linear value for the ith event and n = number of events [129].

These results demonstrated that transfection efficiency of lipopolyplexes in VX2 cells and the resulting GFP protein expression was moderate whereas in case of COS-7 cells, significant GFP expression was observed after flow cytometry analysis exhibiting high transfection efficiency of lipopolyplexes.

Commercially available transfection reagent, Lipofectamine 2000™ was used as a positive control. In case of VX2 cells, although positive control showed slightly higher transfection efficiency than lipopolyplexes but the difference was not significant elucidating the low sensitivity of VX2 cells towards transfectability. COS-7 cells on the other hand, showed significant transfection sensitivity for lipopolyplexes as well as for positive control. Lipopolyplexes showed even better transfection efficiency in COS-7 cells than Lipofectamine 2000™ (figure 6).

Although Lipofectamine 2000™ showed better transfection in VX2 cells, it was found more cytotoxic (figure 8) and also a high-cost product. On contrary, lipopolyplexes exhibited less cytotoxicity (figure 8). In addition, lipopolyplexes preparation is cost effective for the intended use in a wide scheme of experimentation with numerous transfection trials making them the ideal transfection reagents for in-depth assessment of gene therapy approaches.

3.2.2 RT qPCR analysis

RT qPCR assay is widely used for the quantification of transfection efficiencies [130]. The relative quantification assessed by calculating the fold change between two samples allows for accurate insight into the efficiency of a transfection system [131].

In order to quantify the transfection efficiency of lipopolyplexes, mRNA expression of E7_GFP plasmid in VX2 and COS-7 cells was analysed by RT qPCR. Untransfected VX2 and COS-7 cells served as a negative control. In case of both cell lines, cells transfected with Lipofectamine 2000™ were used as positive control as it has been widely used as a transfecting reagent for all cell lines as a non-viral nucleic acid delivery vehicle.

E7 gene is endogenously expressed in VX2 cells, however, in un-transfected VX2 cells, expression of E7 gene is 1 (figure 7), this is because of the fact that untreated VX2 cells are taken as a control and in qPCR fold change calculation of a gene expression, control is always taken as a constant value i.e.1. So, in transfected VX2 cells, fold change above 1 was taken as the transfection efficiency by using lipopolyplexes or Lipofectamine 2000™ (positive control) as transfecting reagents.

In case of COS-7 cells, E7 gene does not exist endogenously. So untreated COS-7 cells are taken as control which is always a constant value for qPCR fold change calculations of a gene expression i.e. 1. Any expression of E7 gene above this constant value of control is taken as the efficiency of transfection.

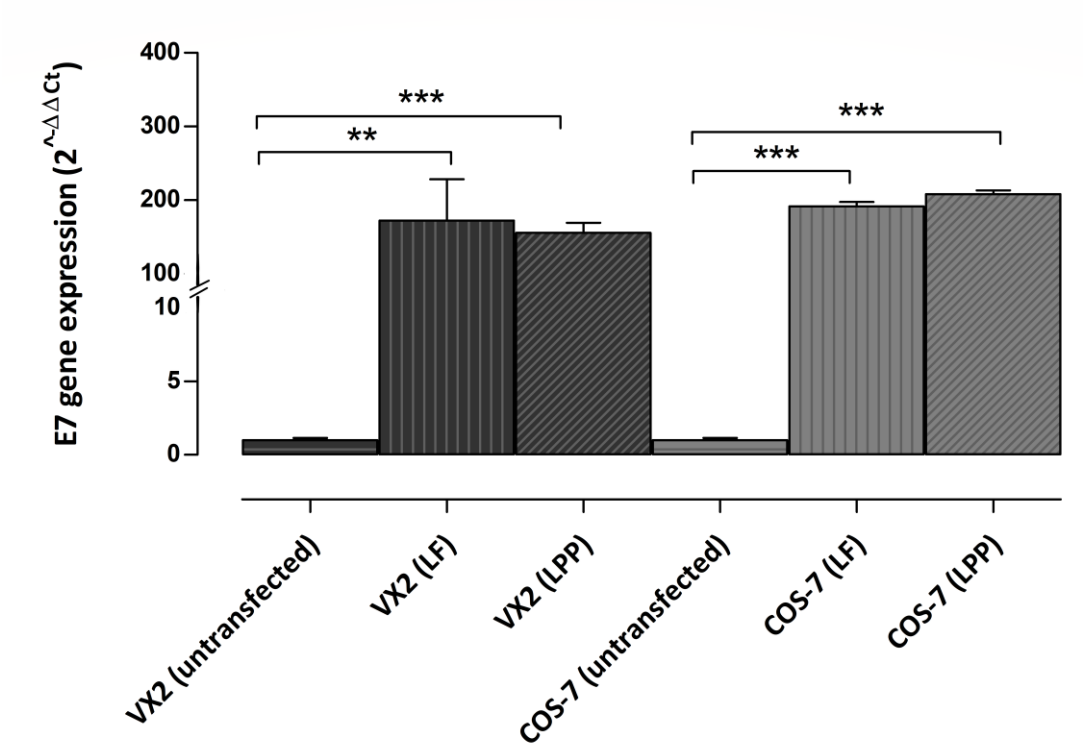

Figure 7. *Quantification of E7_GFP gene expression in VX2 and COS-7 cells after transfection with E7-GFP plasmids using Lipofectamine 2000™ (LF) and lipopolyplexes (LPP)*

RT qPCR analysis revealed considerable transfection efficiency of lipopolyplexes in VX2 and COS-7 which are comparable with the transfection efficiency of commercially available Lipofectamine 2000™. This result is in agreement with the results obtained after flow cytometry analysis for the assessment of lipopolyplex transfection efficiency. E7_GFP expression was calculated by the delta delta Ct method with GAPDH serving as a house-keeping gene to normalize the expression levels of transfected genes.

3.2.3 Cytotoxicity assay

The MTT assay was deployed to quantify cell viability and proliferation. The major limitation to use most of the commercially available transfecting reagents is their higher cytotoxicity whereas lipopolyplexes show less cellular toxicity and thus considered safer [132] than commercially available transfecting reagents such as Lipofectamine 2000™. To evaluate safety of lipopolyplexes as transfecting reagent, MTT dye was added to the cells

and the absorbance of formazan crystals, that form in metabolically active cells, was measured which is directly proportional to the number of viable cells [133].

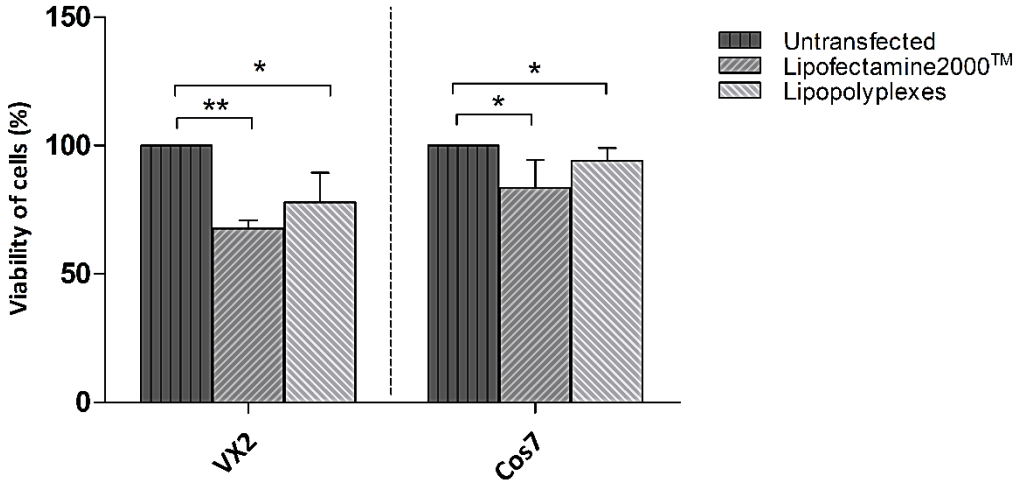

Figure 8. *Cell viability assay of VX2 and COS-7. Lipofectamine 2000™ was used as positive control whereas untreated cells as negative control*

For cytotoxicity evaluation, VX2 and COS-7 cells were transfected with E7_GFP pDNA by using lipopolyplexes as transfecting reagents whereas Lipofectamine 2000™ as positive control for transfecting cells. For all transfections, 1 µg pDNA (E7_GFP) was used. Un-transfected VX2 and COS-7 cells were used as negative control (blank).

Percentage cell viability was measured for both cell lines which showed less percentage of cell viability when Lipofectamine 2000™ was used as transfecting reagent in contrast to lipopolyplexes in both cell lines. Thus, lipopolyplexes were deemed safer transfecting reagents for VX2 and COS-7 cells and found to cause less cellular damage.

Section II

siRNA-mediated knockdown in VX2 cells

3.3 Quantification of E6 and E7 gene expression in VX2 cells

CRPV E6 and E7 genes expression was detected and quantified in VX2 cells by calculating fold changes analysed by RT qPCR. cDNA prepared from rabbit skin was used as negative control whereas cDNA from VX2 tumor tissue was taken as positive control.

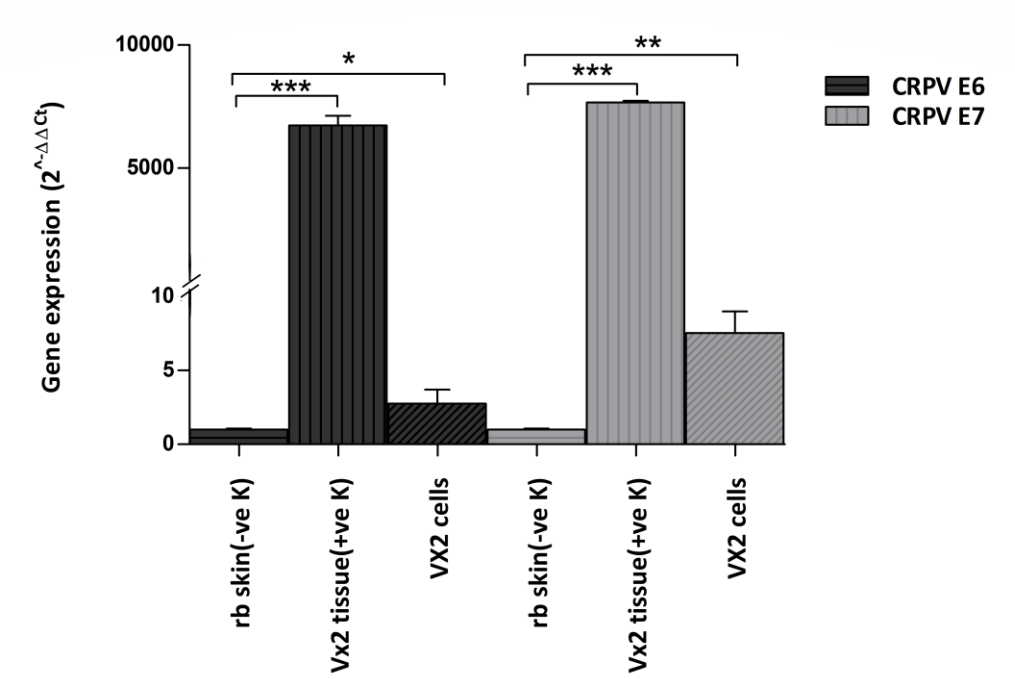

Figure 9. *Detection of CRPV E6 and E7 gene expressions in VX2 cells. GAPDH and RPL32 were used as house-keeping genes*

CRPV E6 and E7 oncogenes were detected in VX2 cells and found in significant quantitative range in comparison to negative control and assumed as suitable *in vitro* imitators of VX2 tumor physiology in order to be used for gene therapy investigations.

3.4 Knockdown of E6 and E7 by siRNA using lipopolyplexes

3.4.1 siRNA Sequences

Three different types of nucleotide compositions for siRNA were selected from genome sequences of CRPV E6 and E7 as shown previously. Interestingly, all individual sequences of both E6 and E7 oncogenes exhibited almost the same knockdown potential. Same was observed when a combination of siRNA sequences was employed, so after initial studies, further siRNA transfection experiments were performed by using randomly any of the sequences from the stock.

3.4.2 Titration of CRPV E6 and E7 siRNA concentrations

Previously reported data indicated roles of CRPV E6 and E7 as transforming genes in papilloma formation and also addressed the importance of these genes as possible therapeutic targets in CRPV induced carcinomas [74]. Downregulation or suppression of either of these genes may cause a significant decline in papilloma development. Titration for selection of a moderate concentration of siRNA with significant knockdown potential yet minimum cytotoxicity is a relevant first step [134]. Keeping that in mind, different concentrations of siRNA starting from as low as 12.5 nmol/L were deployed to evaluate their effect on CRPV E6 and E7 oncogene downregulation (figure 10).

In all experiments, non-target control siRNA was utilized at the same concentration as target E6/E7 specific siRNA. All experiments were performed thrice and the average expression was used to calculate the fold change of CRPV E6 or E7 transcripts. The optimal concentration for knockdown of CRPV E6 and E7 genes was found to be 50 nmol/L.

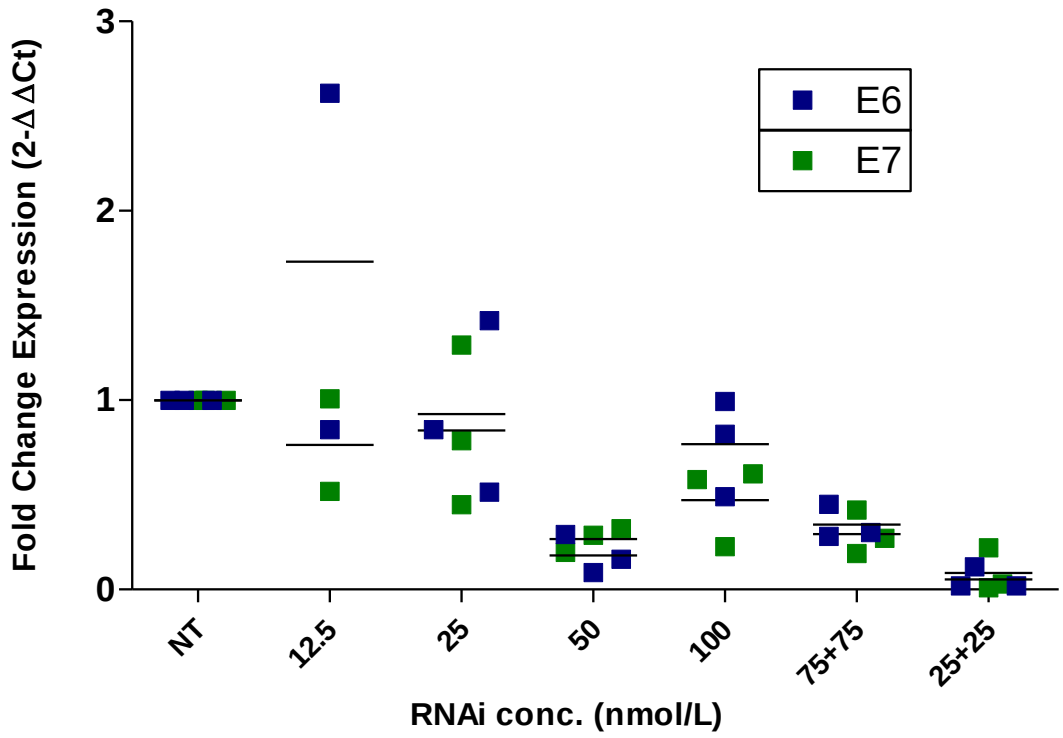

Figure 10. *A collective overview of different concentrations of CRPV E6 and E7 siRNA used for the downregulation of CRPV E6 and E7 oncogenes. Combinations of CRPV E6 and E7 siRNA were also deployed*

A combination of both siRNAs (E6+E7) in a concentration of 25 nmol/L individually, resulted in a dramatically efficient knockdown of both genes. This collaborative effect of siRNA (E6+E7) is witnessed because both of these oncogenes work synergistically as previously found in rabbits (CRPV) as well as in humans (HPV) [135].

Another study reported the outcome of CRPV E1, E2, E6 and E7 vaccination, both individually and in combination, in rabbits previously injected with CRPV viral DNA at specific sites. Papilloma grew in all (100 %) non vaccinated rabbits. Rabbits vaccinated with a single gene vaccine were only partially protected against papilloma growth on the challenged sites. However, out of four rabbits vaccinated with E1, E2, E6 and E7 combination vaccine, two rabbits became completely free of papillomas while in the other two rabbits, only small papillomas developed at the challenged sites which regressed within three weeks. This outcome showed that CRPV oncoproteins, when targeted in combination may lead to synergistic therapeutic effects [136].

These findings also correlate with our findings which demonstrate that CRPV E6 and E7 knockdown shows major effects if siRNAs against E6 and E7 oncogenes are used in combination. It was also observed that less amount of siRNA is sufficient for the knockdown of CRPV E6 and E7 oncogenes if a combination of siRNA is employed.

3.4.3 RT qPCR analysis of selected siRNA concentration

CRPV E6 and E7 gene knockdown at a concentration of 50nmol/L(individually) or 25nmol/L (in combination), are depicted in the figures 10 and 11. All experiments were performed in triplicates.

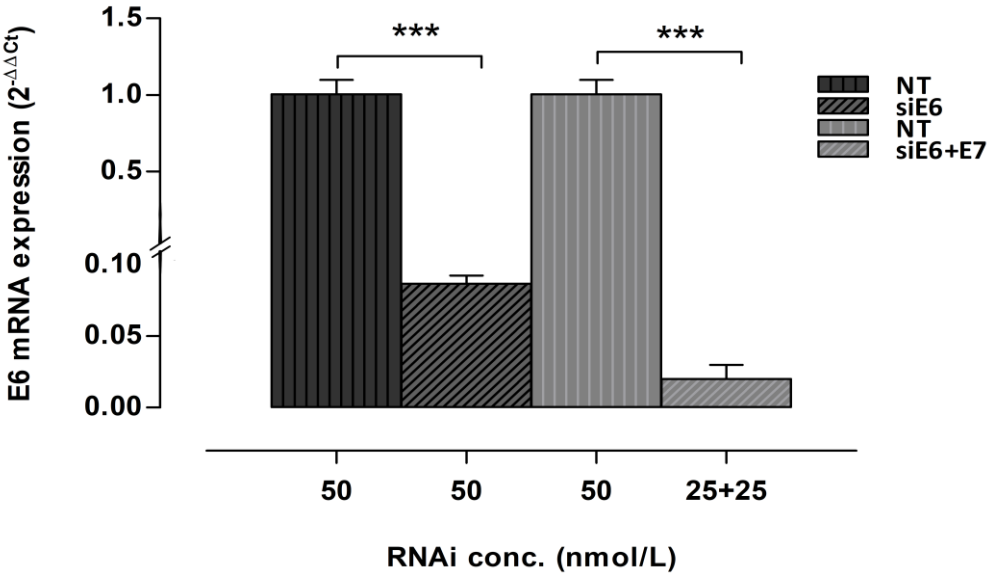

Figure 11. *RT qPCR analysis showing downregulation of CRPV E6 with E6 siRNA alone and as combination of CRPV E6 and E7 siRNA*

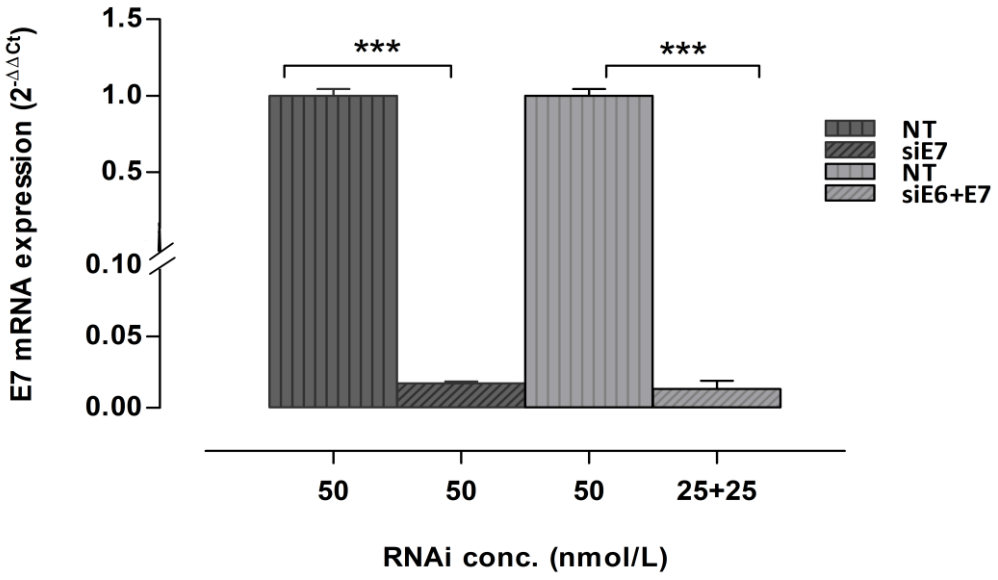

Figure 12. *RT qPCR analysis showing downregulation of CRPV E7 with E7 siRNA individually and as combination of CRPV E6 and E7 siRNA*

Inhibition of CRPV E6 and E7 gene expression was greater than 90 % as a result of siRNA targeting the single gene and even greater than 95 % when targeting both genes.

A lot of factors influence on siRNA-mediated knockdown of a gene such as the choice of siRNA sequence, concentration, transfecting reagent and type of cell line etc. [134]. As this is difficult to transfect 100% of selected concentration of siRNA into the cells without encountering any loss either due to pipetting or transfection efficiency, a considerable volume of siRNA complex from total volume does not reach within the cell, thus we can suggest only an approximate concentration of siRNA useable for knockdown of a gene. For VX2 cells, after working with various concentrations, a concentration of 50 nmol/L for CRPV E6 and E7 oncogenes was found the most suitable for significant downregulation of oncogenes and their relative oncoproteins.

3.4.4 Cell viability analysis

To determine the effect of knockdown of CRPV E6 and E7 oncogenes on the rate of proliferation and viability of VX2 cells, MTT viability assay was employed.

As previous studies demonstrated the role of CRPV E6 in protecting tumor cells from apoptosis and maintaining cancer cell population integrity [137], this is also observed in VX2 tumor cells. The percentage of viable VX2 cells was reduced considerably after inhibition of CRPV E6 and E7 genes by siRNA (figure 13).

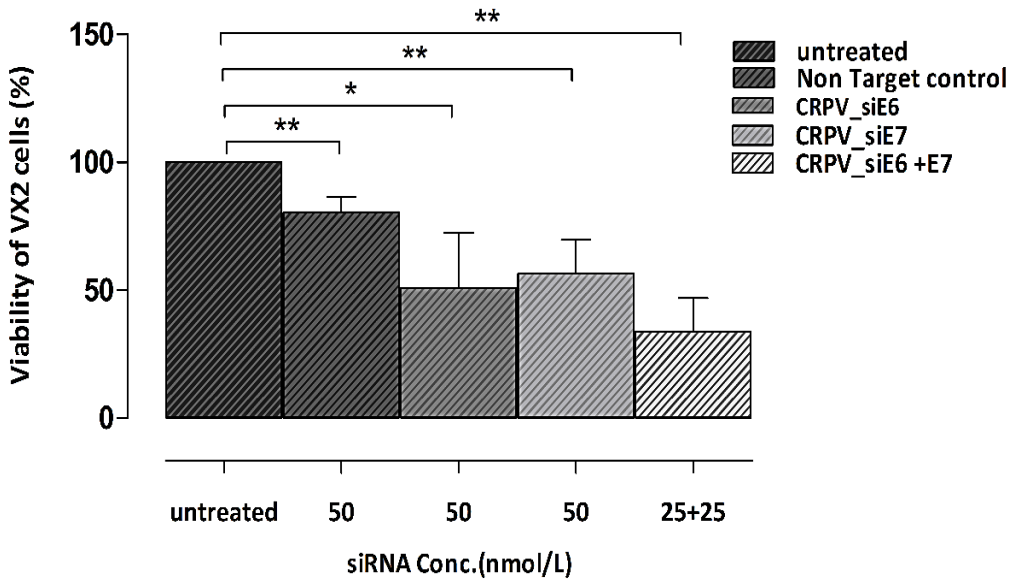

Figure 13. *Cell viability of VX2 cells (MTT) after siRNA-mediated knockdown*

Various studies have shown previously that CRPV E6 and E7 oncogenes play a vital role in the transformation and anchorage independent growth of tumor cells [138]. siRNA-mediated downregulation of these genes certainly puts a halt on the rate of proliferation of CRPV positive cells as shown in figure 13. A combined use of E6 + E7 siRNA decreased proliferation rate of VX2 cells which is in accordance with the synergistic effect resulting in higher knockdown of these genes on transcript level as exhibited in previous data shown in RT qPCR results (figure 11 and 12)

3.4.5 Western blot analysis

PCNA is used as one of the most reliable proliferation markers in tumors [139]. Western blot analysis was carried out to analyse downregulation of PCNA (proliferating cell nuclear antigen) which is responsible for regulating the process of DNA replication during

proliferation of cells. PCNA protein has a vital role in cellular physiology by taking part in DNA replication and repair regulatory system [140].

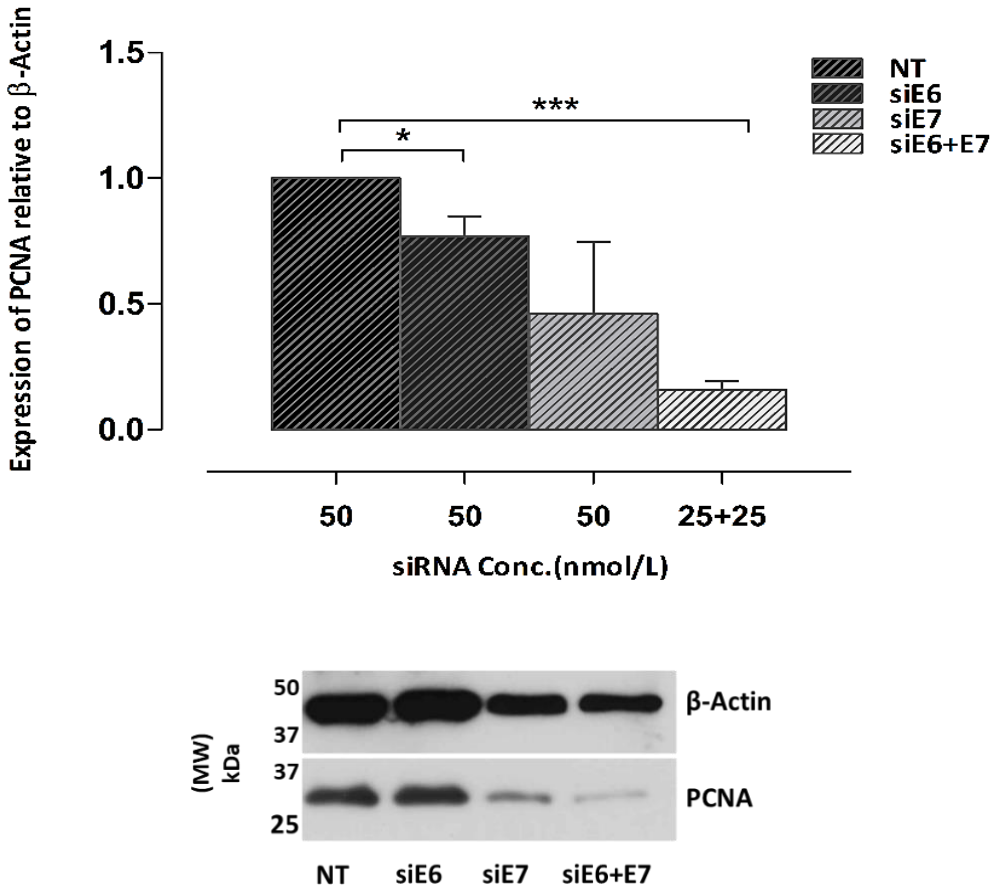

Figure 14. *Western blot analysis to analyse expression of PCNA in VX2 cells after siRNA knockdown by using CRPV_E6, E7 and E6+E7*

Since there are no commercially available antibodies for the detection of CRPV E6 and E7, the influence of siRNA knockdown on VX2 cells proliferation was indirectly evaluated in western blot. Beta actin was used as a reference protein for the normalization of PCNA expression. Three individual experiments were carried out and average expression of PCNA was calculated.

Significant downregulation of PCNA protein expression was observed in VX2 cells after CRPV E6 and E7 siRNA-mediated knockdown (figure 14) which indicated less rate of proliferation of cells after siRNA treatment. CRPV E7 knockdown alone showed variable

effects on PCNA expression (not statistically significant). Non-target small RNA treated VX2 cells were used as a control. Protein bands were quantified on the basis of optical density/intensity by ImageJ/Fiji software [141]. Western blot analysis of siRNA (E6+E7) treated cells showed prominent decline in PCNA protein levels which suggested significantly less proliferation rate due to downregulation of CRPV E6 and E7 oncogenes. These results are in accordance with the previous outcomes demonstrated in figure 11, 12 and 13.

3.4.6 Cell cycle analysis of VX2 cells after CRPV E6 and E7 knockdown

Cell cycle comprises of a combination of a lot of metabolic changes in dividing cells. There are four phases of cell cycle: (a) G1 is a postmitotic phase in which cell synthesizes RNA and proteins, (b) S phase, DNA doubles, RNA and proteins continue to be produced, (c) G2, no new DNA synthesis whereas RNA and proteins still generated (d) mitosis, completion of cell division [142].

It has been previously shown that VX2 cells show major distribution of cells in G1 phase 69.3%, 5.6% in G2 phase and 25.1% in S phase. Time of population doubling was found approximately 34.5 hours [83]. In VX2 cells, G1 phase was found to be 53%, G2 phase 5% whereas S phase was 42% which suggests G1 as the most prominent phase in these cells closely followed by S phase.

This data also affirms VX2 cells as highly proliferative as G1 and S phases constitute mainly of reproductive events of the cells and both of these phases cover almost the whole cycle of VX2 cells. These results of cell cycle distribution in VX2 cells are in accordance with the previous findings [83].

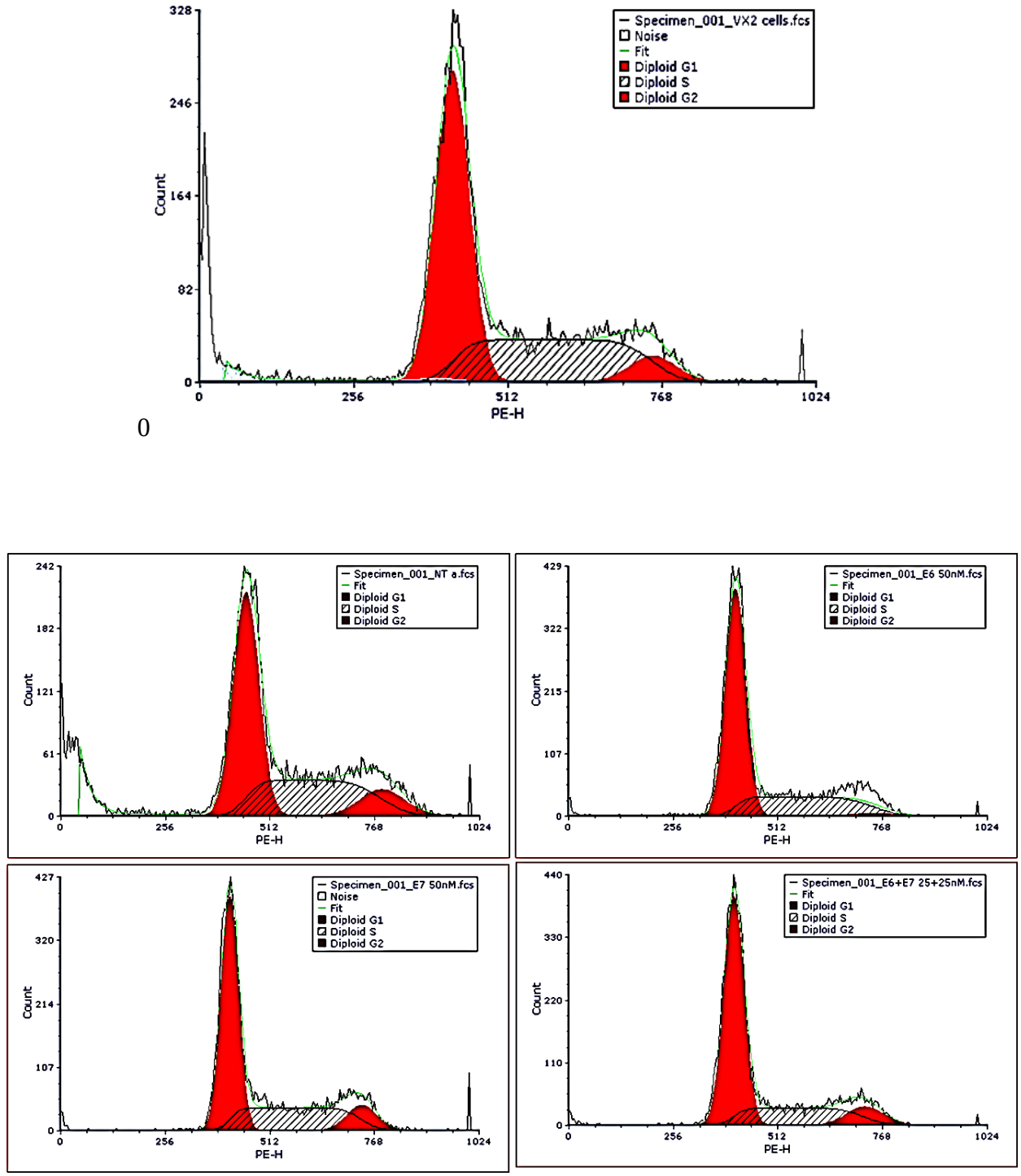

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Figure 15. *Cell cycle phases distribution of VX2 cells analysed after treatment with siRNAs: CRPV_siE6 50 nmol/L, CRPV_siE7 5 0nmol/L and CRPV_siE6+E7 (25+25) nmol/L*

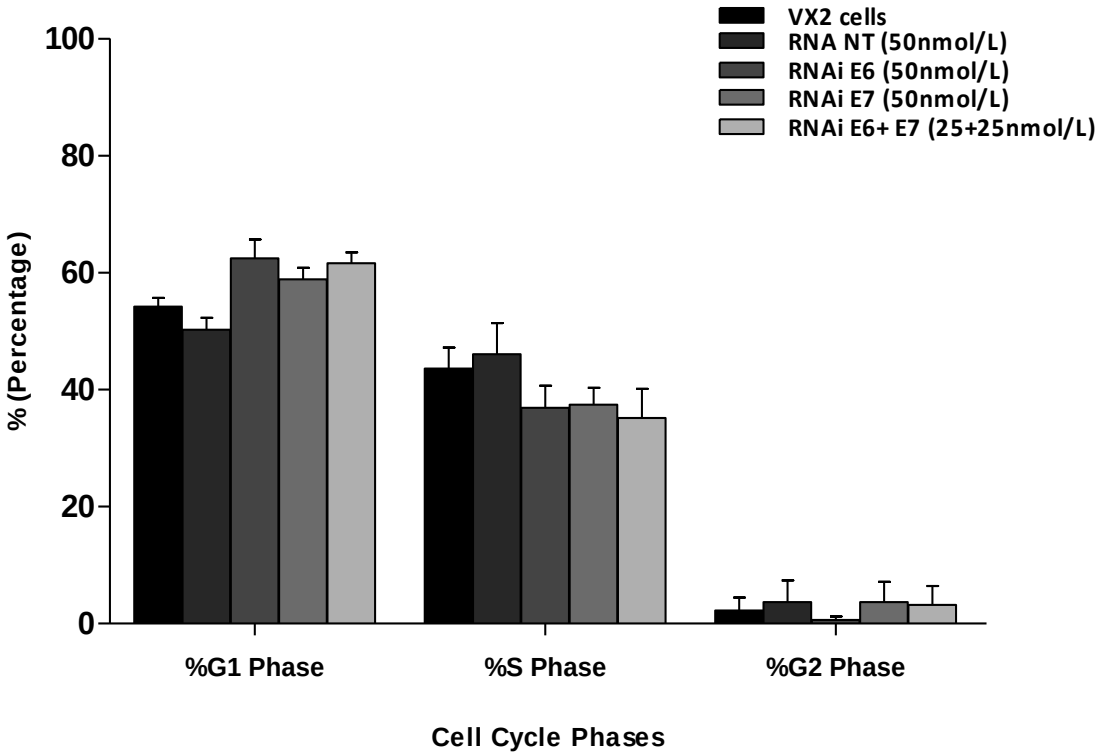

Figure 16. *Percentage of VX2 cells in G0/G1 phase and S phases post downregulation of CRPV oncogenes*

Effect of siRNA treatment and resulting downregulation of CRPV E6 and E7 oncogenes showed an arrest in S phase which is DNA replication phase while upregulation of G1 phase. G2 phase remained least perturbed as a result of siRNA transfection in VX2 cells. A decline in S phase shows that division of cells into daughter cells was affected after siRNA transfection. This finding is in accordance with previously shown data and suggests a decrease in cell proliferation rate of VX2 cells as a result of knockdown of CRPV E6 and E7 oncogenes, whether used individually or in combination. All experiments were performed in triplicates and values shown (figure 16) were calculated as an average of values of all experiments for cell cycle analysis.

3.4.7 Wound healing assay

Previously, it was found that knockdown of E6 and E7 alone or in combination elicits an effect on the rate of cell migration *in vitro* in cervical cancers such as Caski and SiHa cells [143]. To evaluate the influence of CRPV E6 and E7 on cell migration in VX2 cells, wound

healing assay was performed. Cell migration analysis revealed that 24 h after scratch induction in a VX2 cells layer, the wound closure reduced after E6/E7 siRNA knockdown (figure 17).

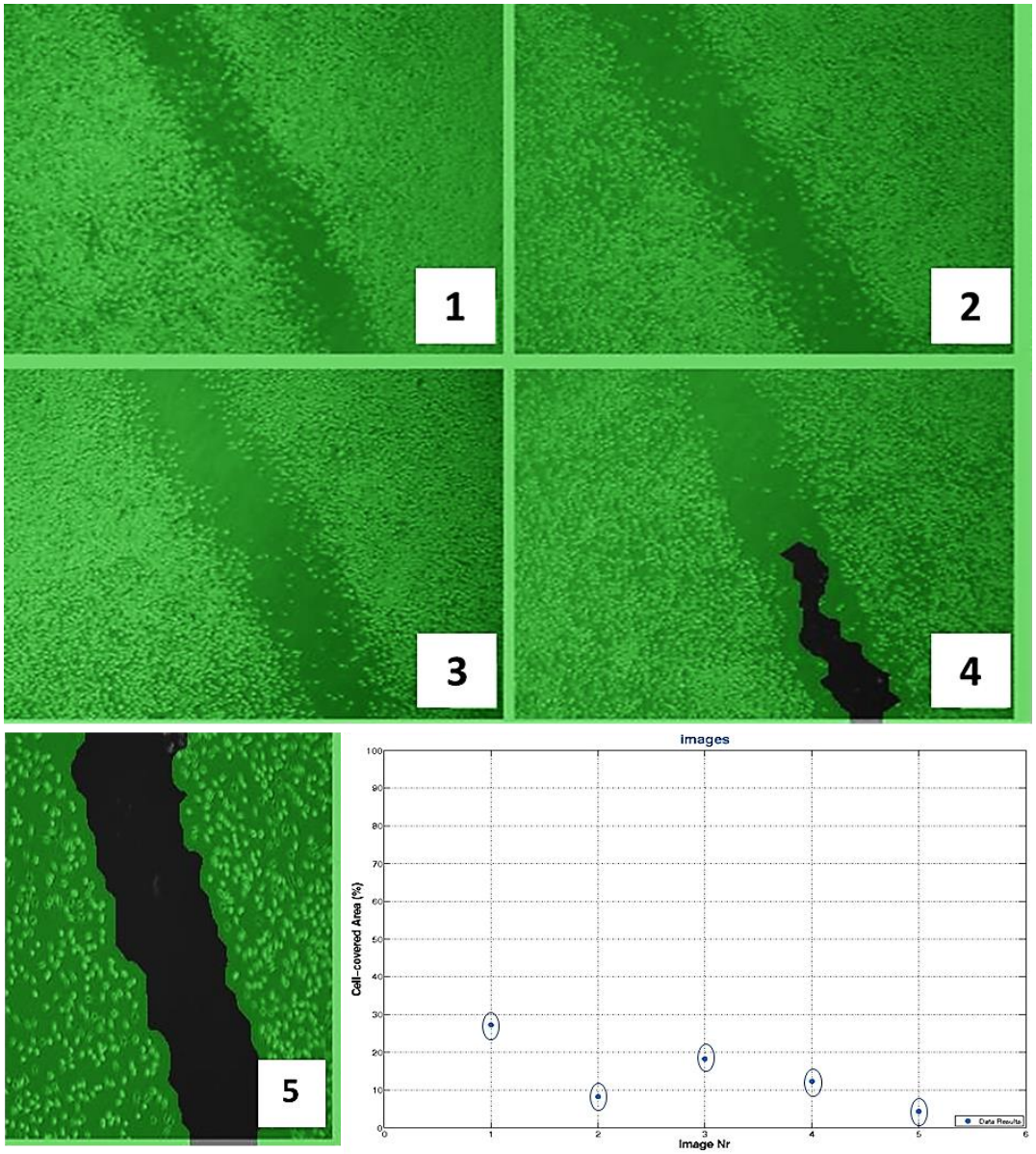

Figure 17. *Images 1, 2, 3 and 4 show VX2 cells with scratch induction after 24 hrs, Image 1: migration of NT small RNA (control) treated cells, Image 2: cells treated with siE6, Image 3: siE7 treated cells, Image 4: siE6+E7 treated cells, Image 5 displays an average of %age cell-covered area in all the wells after inducing a scratch (at zero time)*

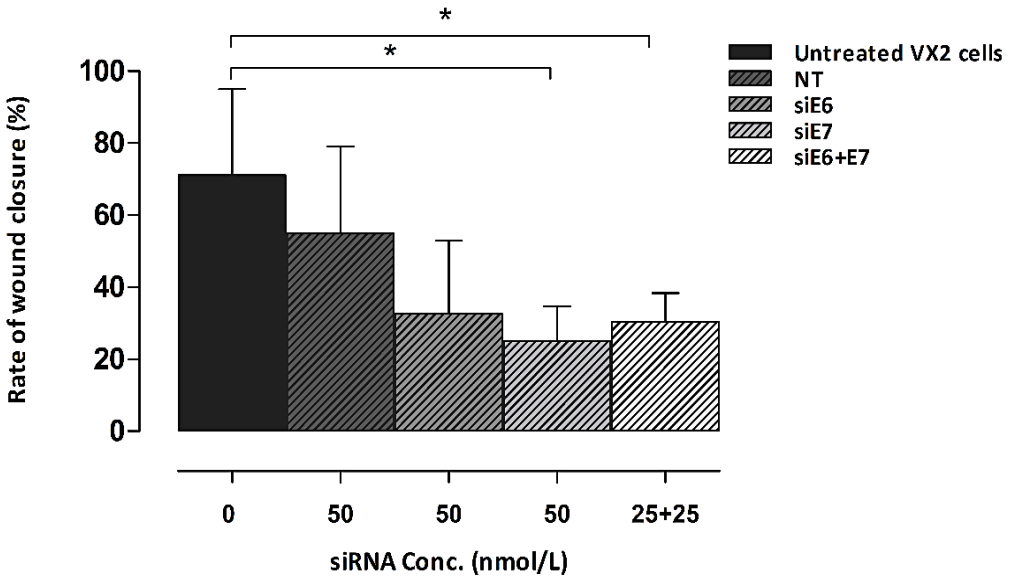

Figure 18. *Rate of wound healing after 24 h in VX2 cells previously transfected by siRNA against CRPV E6 and E7 genes, individually and in combination*

Rate of wound healing showed less movement of VX2 cells towards wound closure as compared to VX2 cells treated with non-target siRNA. Downregulation of CRPV E6 and E7 genes, individually or combined appeared to inhibit cell migration in VX2 cells. All experiments were performed thrice and analysed by Imagej/Fiji [141] and Wimasis Microscopy Image Analysis software for visual representation.

3.4.8 Branching morphogenesis microscopy analysis

The basis of branching morphogenesis originates from the ability of epithelial cells or endothelial sheaths to remodel themselves into tubular networks which ultimately develops into different organs. This phenomena can be observed *in vitro* when co-culturing epithelial cells with fibroblasts [144]. Epithelial branching morphogenesis is a heavily co-ordinated and synchronized system which facilitates cell proliferation, cell to cell communication and cell matrix interaction which is a prerequisite for organogenesis. Among all characteristics that accompany this process, cell to cell communication and cell matrix interaction were evaluated in VX2 cells after CRPV E6 and E7 knockdown.

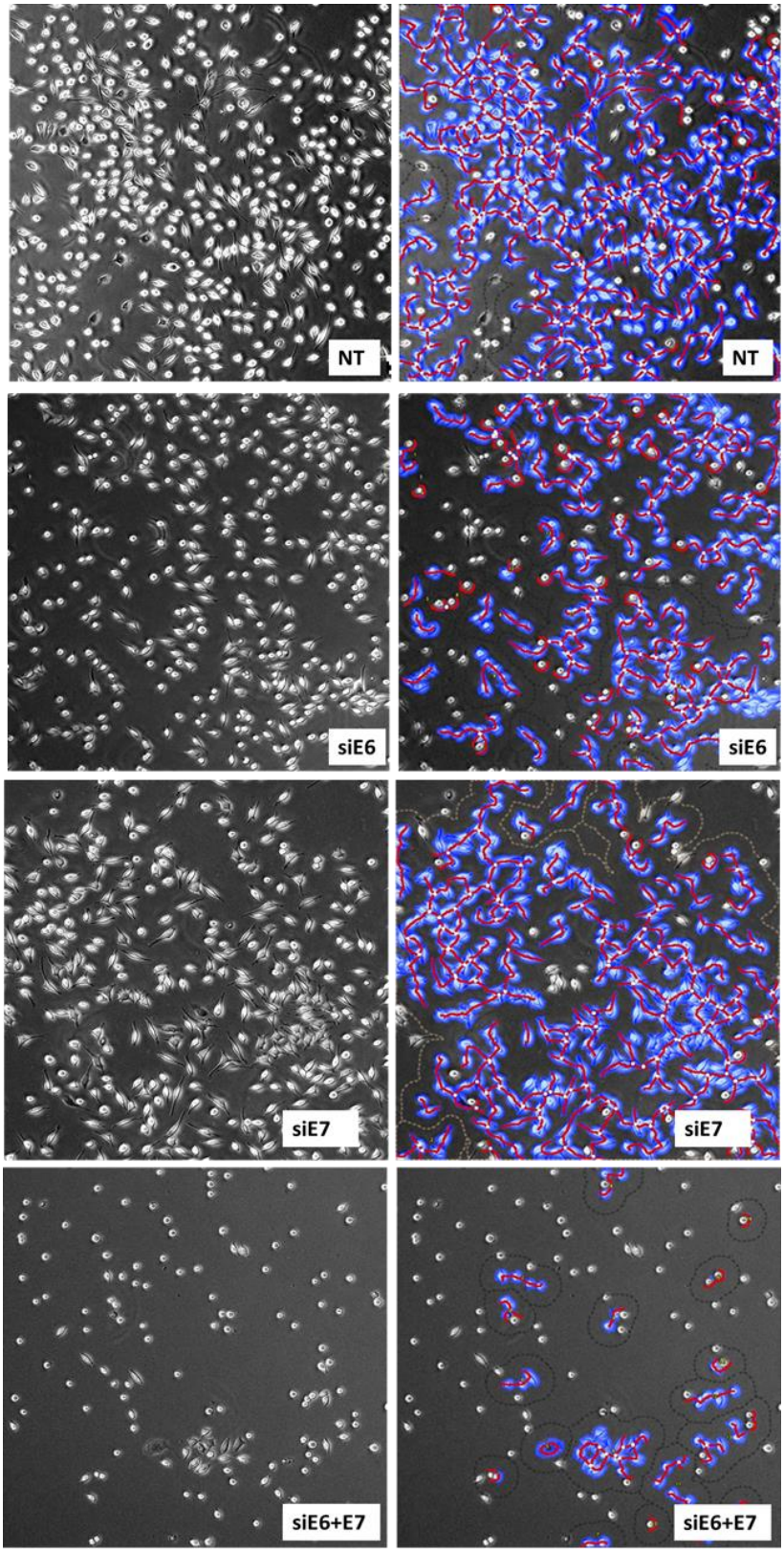

Figure 19. *Microscopic view (20x) of phenotypic differences between VX2 cells without and with connected branching points*

24 h after siRNA treatment, morphology of VX2 cells was observed under the microscope and images were captured for subsequent analysis.

Branching points between VX2 cells treated with siRNA E6, siRNA E7 and their combination (siRNA E6+E7) were counted with the Wimasis Microscopy analysis software. Nontarget small RNA treated VX2 cells were taken as a control. Results expressed a decrease in the number of branching points after E6/E7 knockdown (figure 19).

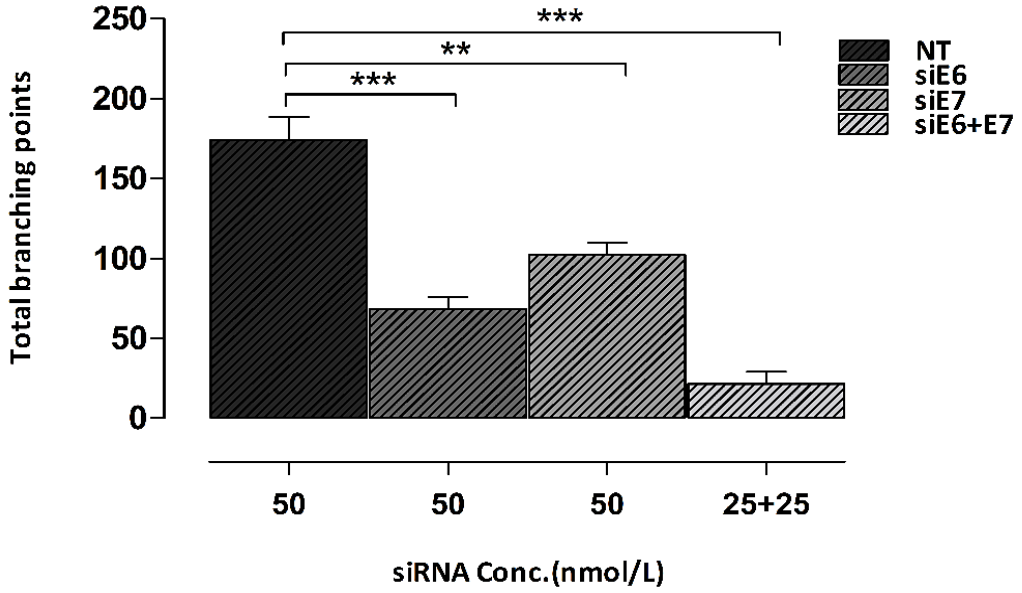

Figure 20. *Quantification of phenotypic differences on the basis of number of branching points between adjacent VX2 cells after siRNA-mediated oncogenes knockdown*

A considerable decline in the number of branching points between adjacent VX2 cells was observed after E6 oncogene knockdown. Although branching connection points were less in VX2 cells treated with E7 siRNA as compared to control nontarget (NT) siRNA treated cells, this effect was less in those cells treated with siRNA E6 alone or siRNA E6+E7. This shows that CRPV E6 downregulation affects greatly on cell phenotype and morphology. Moreover, rate of cell proliferation decreases drastically after using siRNA combination of E6 and E7 on VX2 cells, thus fewer branching points were observed between adjacent cells because of reduced number of cells.

This assay enables us to quantify the number of proliferation rate of cells without exercising any kind of lysis or disturbance to the cells and while letting the cells keep growing during the investigation of effect of treatment on their phenotype and cell number. This method is very simple, convenient and can be used to document the apparent changes in cells treated with control and treatment formulations at various time points under microscope.

3.4.9 Real time cellular analysis

Cellular motility and adhesion are integral mechanisms behind metastasis and rapid tumor growth [145]. xCELLigence system is used to monitor live cell viability, proliferation, motility, adhesion, migration, invasion, cell number and morphology. It provides label free and real-time surveillance of cell viability by tracking the electrical impedance as a readout [146]. xCELLigence system prevents hands-on manipulation of cells thus leading to better physiologically relevant results. Moreover, labelling or dying of cells for migration and invasion assays can lead to changed gene profiles resulting in altered results and to avoid this, xCELLigence system provides kinetic analysis by recording entire processes related to cells mobility in real-time [147].

Real time cellular analysis was performed with the xCELLigence system to measure cell viability in VX2 cells after siRNA-mediated knockdown of CRPV E6. For real time monitoring of cell growth and assessment of cell proliferation at different time points after receiving siRNA treatment, VX2 cells were grown for 24 h onto specifically designed E-plates. The first peak on the electrical impedance graph (figure 21) shows the time point when VX2 cells were added into wells of the E-Plate. Second peak represents the time point (24 h after addition of cells) when (non-target and E6) siRNA loaded lipopolyplexes were added to the cells. VX2 cells were subsequently monitored for 72 h and their proliferation rate was documented. Non-target siRNA treated VX2 cells were used as a control.

It was observed that VX2 cells showed a slumped proliferation rate after siRNA transfection and this trend persisted for the entire length of electrical impedance recording (72 h). This outcome suggested a decrease in the cell number, rate of growth, invasion and motility of VX2 cells and thus invariably reduced growth of CRPV positive cells after CRPV E6 gene was downregulated because of siRNA-mediated targeting.

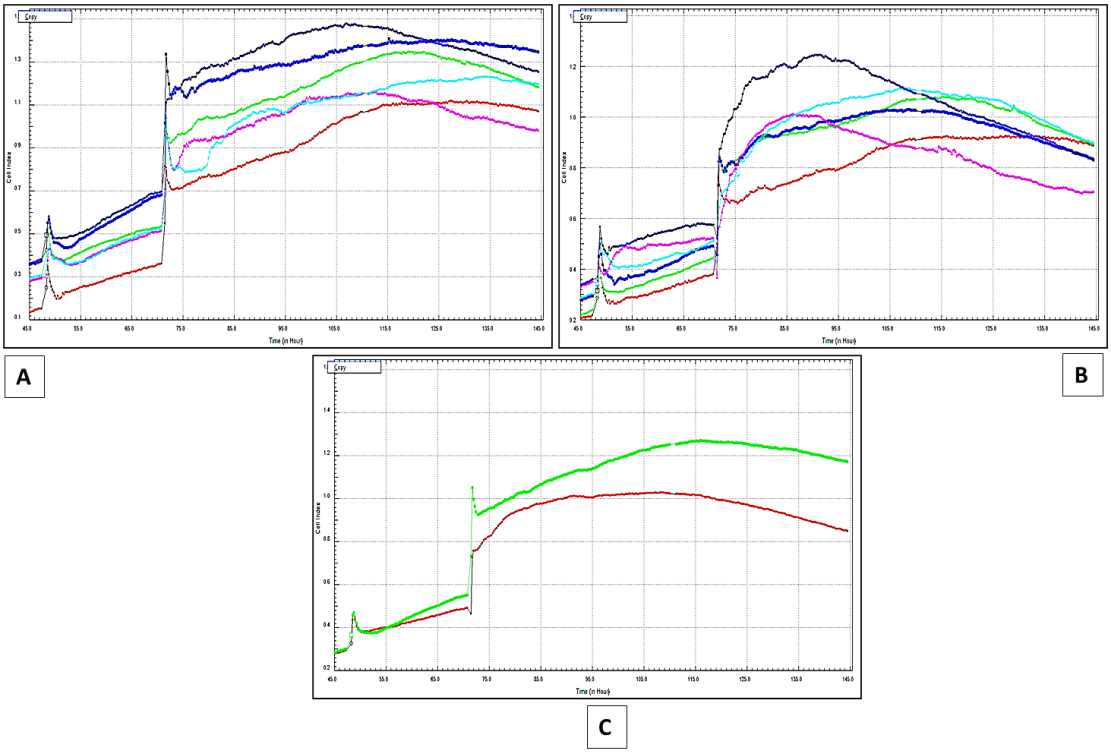

Figure 21. *Real time cellular analysis of proliferation rate of VX2 cells after CRPV E6 knockdown. (A) cells treated with NT control RNA (50 nmol/L) (Each line represents one well of a 96 well E-plate). (B) cells treated with siE6 (50 nmol/L) (C) average of proliferation rates of VX2 cells treated with NT siRNA (green line) against cells treated with E6 siRNA (red line)*

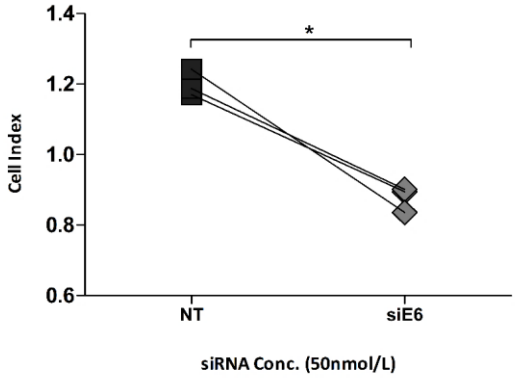

Figure 22. *Cell index depicting proliferation rate of VX2 cells post transfection by non-target siRNA and CRPV E6 siRNA on the basis of TEER measurement*

3.5 Knockdown of CRPV E6 and E7 oncogenes in VX2 tumor suspension cells

Another exceptional method to validate siRNA-mediated downregulation of CRPV oncogenes was the direct use of VX2 tumor suspension. VX2 cells taken directly out of VX2 tumor without being cultured or passaged *in vitro* for longer durations so that these cells possessed all the exact genomic pattern and tumorigenic characteristics that existed within VX2 tumor without undergoing any mutations induced as a result of long-term *in vitro* survival of tumor cells. In this study, VX2 tumor cell suspension was directly subjected to transfection by different concentrations of siRNA targeting CRPV E6 and E7 by using lipopolyplexes as transfecting reagents. RT qPCR analysis was performed on VX2 tumor suspension cells post siRNA transfection after a duration of 48 h incubation, targeting CRPV E6 and E7 oncogenes. Statistically significant downregulation of CRPV E6 gene was observed at 25, 50 and 150 nmol/L siRNA E6 concentrations, where VX2 tumor suspension cells treated with same respective concentrations of non-target siRNA were used as control (figure 23).

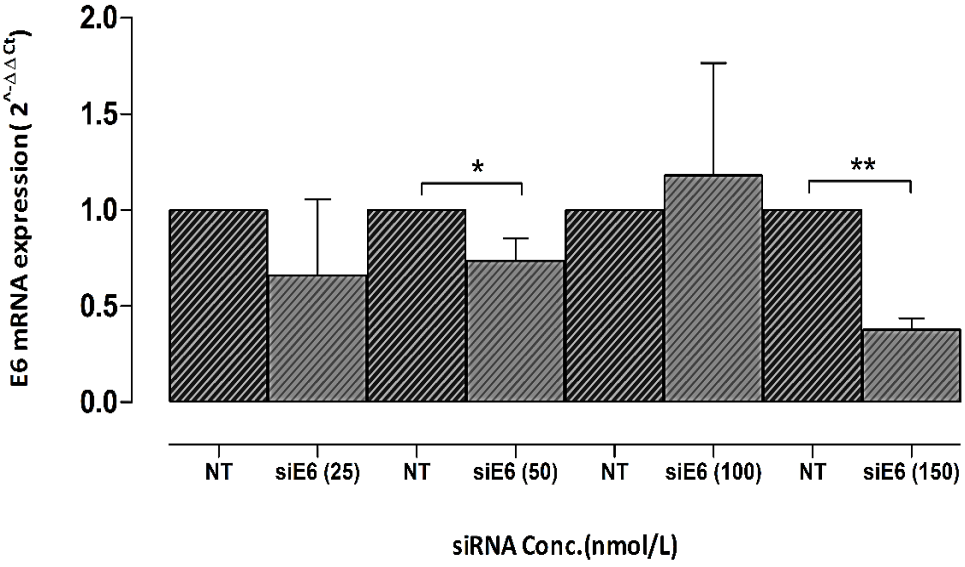

Figure 23. *A collective overview of RT qPCR analysis of different concentrations of siRNA CRPV_E6 used for the downregulation of CRPV E6 oncogene in VX2 tumor cell suspension*

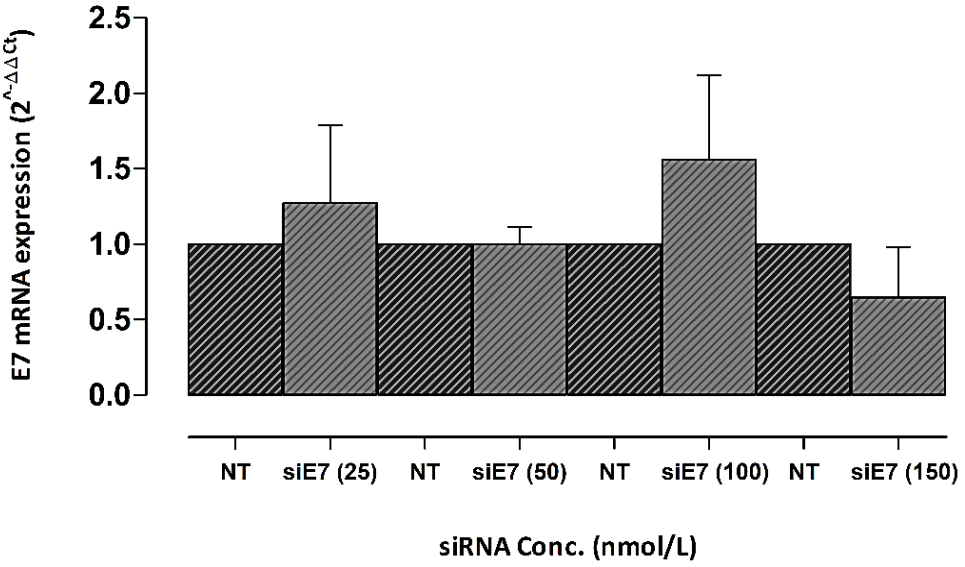

Figure 24. *RT qPCR analysis of different concentrations of siRNA CRPV_E7 after downregulation of E7 oncogene in VX2 tumor cell suspension*

However, in case of different concentrations of siRNA E7 treatment on VX2 tumor suspension cells, statistical significance could not be achieved because of varying Ct values between three individual experiments although at a concentration 150 nmol/L siRNA E7, downregulation of oncogene could be observed (figure 24)

This experiment design was devised only to have a perception about the efficacy of siRNA treatment on raw VX2 tumor tissue although it has various limiting factors such as presence of cellular debris as a result of VX2 tumor trituration in spite of filtration, combination of cancerous and non-cancerous cells in a whole VX2 tissue suspension, non-ideal condition for the cells to overcome adaption/acclimatization shock and thus upregulation or downregulation of various cellular pathways etc. Thus, the titration process to find moderate siRNA concentration for knockdown of CRPV E6 and E7 genes on VX2 tumor cell suspension exhibited mixed character of upregulation and downregulation of these oncogenes unlike a clean culture of only VX2 cells *in-vitro*.

Section III

Exogenous knockdown of CRPV E6 and E7 oncogenes by siRNA loaded lipopolyplexes

3.6 siRNA-mediated knockdown of CRPV E6 and E7 in pre-transfected COS-7 cells

This series of experiments involving a CRPV negative cell line COS-7, was designed for exogenous knockdown of CRPV E6 and E7 oncogenes to create an alternative system if there is no available system for endogenous knockdown of in-house genes of CRPV E6 and E7 and for *in vitro* investigation of these oncogenes. Here, we could confirm the specificity of our previously used siRNAs directed against CRPV E6 and E7 oncogenes. Plasmids encoding CRPV E6 and E7 in the context of a GFP-recombinant protein were previously generated in our lab. The transfection efficiency of the lipopolyplexes was previously verified in COS-7 cells (figure 5 and 7).

Successful transient transfection of the GFP tagged CRPV E6 and E7 encoding plasmids was achieved by using low toxicity lipopolyplexes as transfecting reagents. After verification of GFP expression, COS-7 cells were re-transfected by siRNAs directed against CRPV E6 and E7 and knockdown of these oncogenes was verified by following assays.

3.6.1 Flow cytometry analysis

Previously, green fluorescent protein (GFP) has been used as a fluorescent reporter to quantify gene expression using flow cytometry after performing transfection [148].

In present study, this method has been used to assess the efficiency of siRNA knockdown against transiently transfected CRPV E6 and E7 plasmids by measuring GFP fluorescence using flow cytometry. siRNA directed against CRPV E6 and E7 was deployed to evaluate knockdown on E6 and E7 target genes. COS-7 cells were incubated for 48 h after siRNA treatment followed by detection of GFP fluorescence.

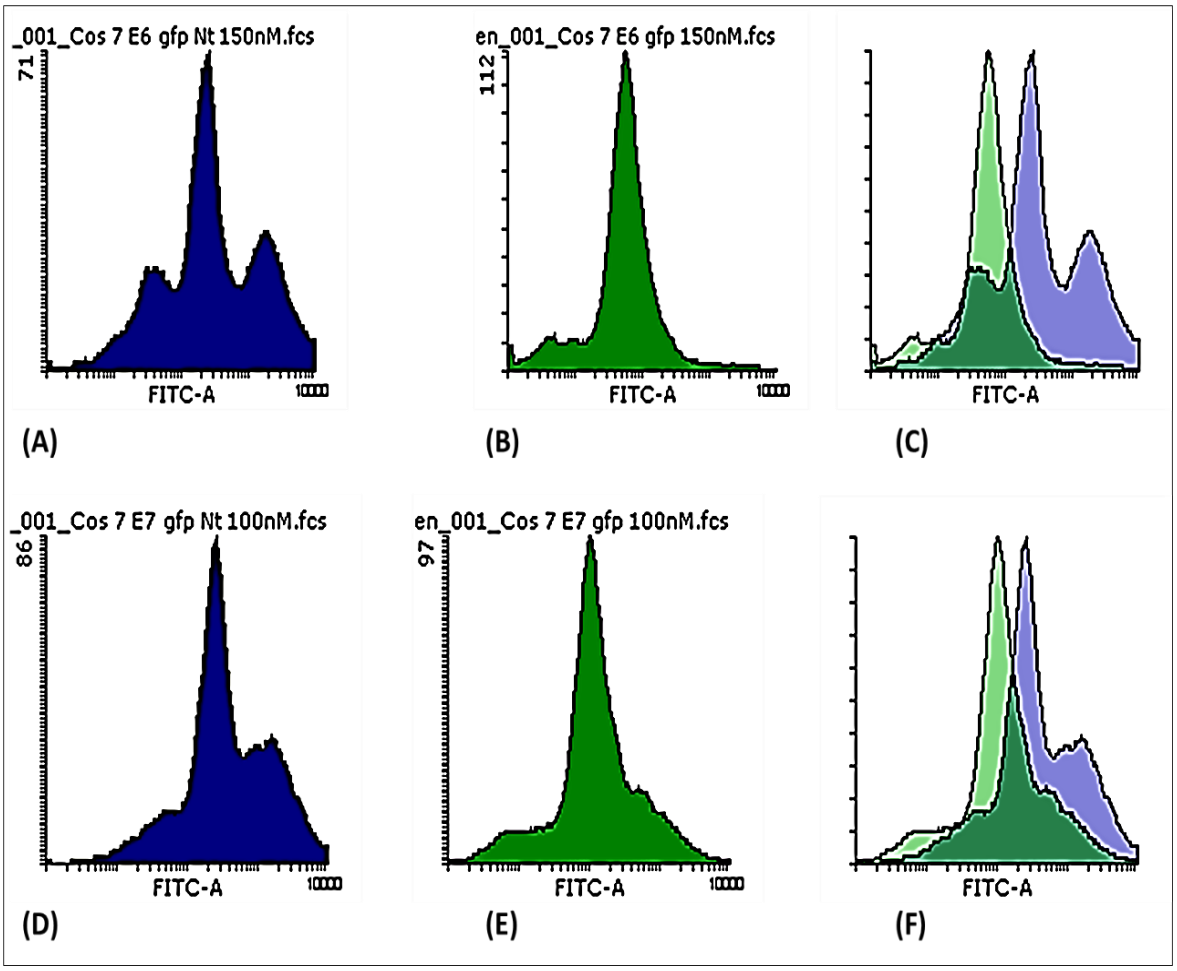

Figure 25. *Histograms of flow cytometry assay expressing GFP positive COS-7 cells. (A, B, C, D, E and F), COS-7 cells previously transfected by GFP tagged CRPV E6/ E7 plasmids. A and D) COS-7 cells re-transfected by 100 and 150 nmol/L Non-target siRNA respectively. B and E) COS-7 cells re-transfected by 150 and 100 nmol/L CRPV_E6 and E7 siRNA respectively. C) overlaying histogram shows a comparison between COS-7 cells transfected with non-target siRNA and CRPV_E6 siRNA. F) overlaying histogram represents COS-7 cells transfected with non-target siRNA and CRPV_E7 siRNA*

FACS analysis demonstrated a decline in GFP fluorescence after siRNA knockdown of CRPV E6 (150 nmol/L) and CRPV E7 (100 nmol/L). COS-7 cells treated with non-target (NT) siRNA at the same concentration were used as a negative control.

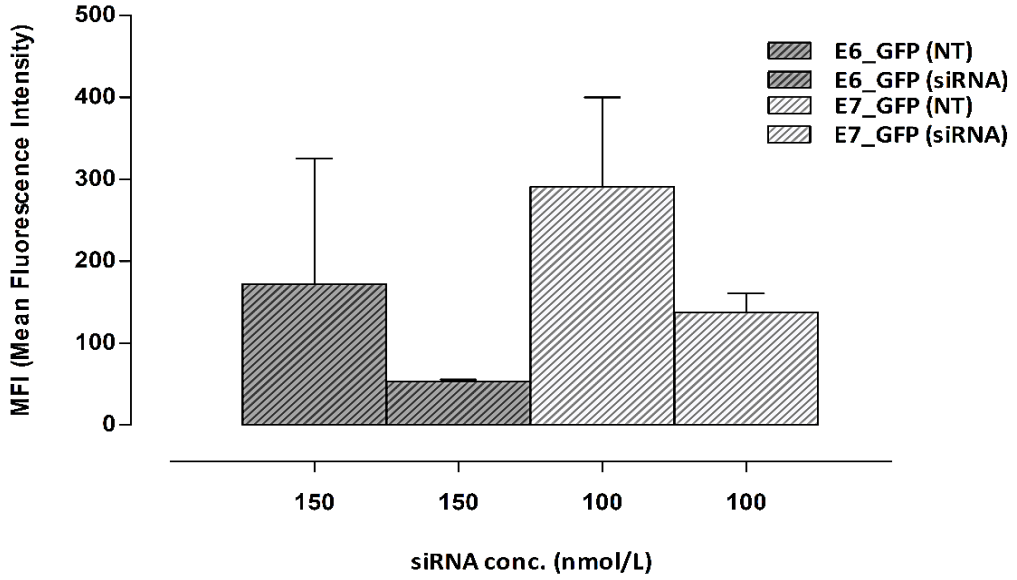

Figure 26. *Mean fluorescence intensity in GFP positive COS-7 cells treated with CRPV oncogenes specific siRNAs*

Fluorescence intensity of GFP was calculated as geometric mean by the formula given below:

$$\text{G-mean}=10^{\Sigma \log X(i)/n}$$

where $X(i)$ is the channel or linear value for the ith event and n is the number of events

The siRNA concentrations used were far higher than siRNA concentrations used previously for the knockdown of E6 and E7 oncogenes in VX2 cells because of the fact that in case of COS-7 cells, after transient transfection, the levels of CRPV E6 and E7 transcripts were found much higher than the number of endogenous transcripts present in VX2 cells. E6 and E7 genes are expressed endogenously in VX2 cells. Moreover, in VX2, CRPV E6 and E7 genes coexist and synergise each other's effect so their efficient knockdown occurs at similar concentrations of siRNA (50 nmol/L), while in COS-7 cells, these genes are expressed exogenously as a result of transient transfection, so both genes do not necessarily downregulate at the same concentration of siRNA.

3.6.2 RT qPCR analysis

CRPV E6_GFP and E7_GFP encoding plasmids were transfected in COS-7 cells and GFP expression was examined after 24 h by fluorescence microscopy. After expression of GFP tagged plasmid was confirmed by fluorescence microscopy, COS-7 cells were transfected with siRNA directed against CRPV E6 and E7 at concentrations of 150 nmol/L and 100 nmol/L. Non-target siRNA was used as control.

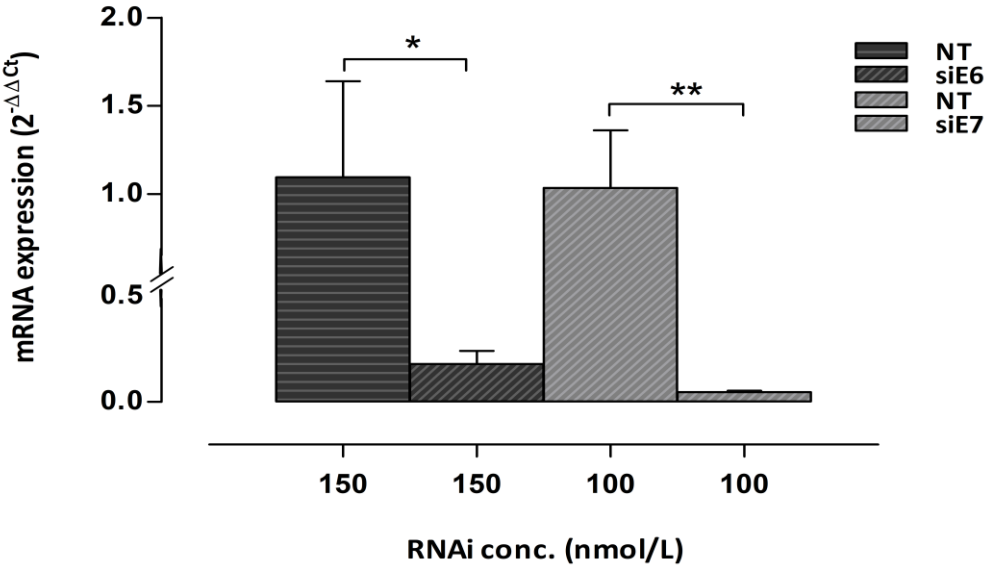

Figure 27. *RT qPCR analysis of siRNA CRPV_E6 and E7 at a concentration of 150 and 100 nmol/L in previously transfected COS-7 cells with CRPV E6 and E7 plasmids*

Real time qPCR showed knockdown of previously transfected CRPV E6 and E7 genes in COS-7 cells by using siRNA loaded lipopolyplexes. This outcome attests the efficiency of siRNA against CRPV oncogenes and eradicates the possibility of abundant off-target effects. Ct values of COS-7 cells transfected with CRPV E6_GFP or E7_GFP plasmids range from18 to 20 cycles which manifest high level of transfection by lipopolyplexes. As identical levels of transfection for both types of plasmids (E6 and E7) are not possible to achieve, so siRNA concentrations to downregulate respective gene after transient transfection also differ than one another i.e. 150 nmol/L for CRPV E6 and 100 nmol/L for CRPV E7.

Ct values of VX2 cells to express expression levels of CRPV E6 and E7 onocogenes are found between 22 to 28 cycles which show lesser number of CRPV E6 and E7 transcripts

than those introduced in COS-7 cells exogenously as a result of transient transfection. Therefore, siRNA concentration for the knockdown of CRPV oncogenes in VX2 cells is less than in COS-7 cells.

3.6.3 Western blot analysis

CRPV E6 and E7 proteins cannot be directly detected by western blot because of non-availability of antibodies against these genes commercially. So, following induction of downregulation of these proteins, a functional protein analysis was carried out [149] in order to see how lower levels of these oncogenes affect different cellular functions of cancer cells which are regulated by these oncoproteins (figure 14).

An alternative approach to directly analyse level of these oncoproteins as a result of gene knockdown is, to introduce sequence of a tag or label such as GFP into the genes of CRPV E6 and E7, transfecting these constructed plasmids into cells and post siRNA treatment using antibody against GFP for the detection of tagged protein in western blot as described by previous likewise protocols [150] .

To analyse downregulation of CRPV E6 and E7 proteins in western blot assay, COS-7 cells were transfected with GFP tagged plasmid DNA of CRPV E6 and E7 in separate 6 well plates. After 24 h incubation followed by confirmation of GFP tagged plasmids uptake under a fluorescent microscope, COS-7 cells were re-transfected with siRNA directed against CRPV E6 in a concentration of 150 nmol/L and with siRNA against CRPV E7 in a concentration of 100 nmol/L (knockdown by using these concentrations of siRNAs was previously established by RT qPCR and flow cytometry analysis) in respective 6 well plates.

Western blot analysis was initiated 48 h post siRNA treatment. In order to estimate the level of CRPV E6 and E7 proteins in COS-7 after siRNA-mediated knockdown, anti-GFP antibody was used as both of these genes were tagged with GFP label. Beta tubulin was used as a house keeping control protein to normalize expression of target proteins.

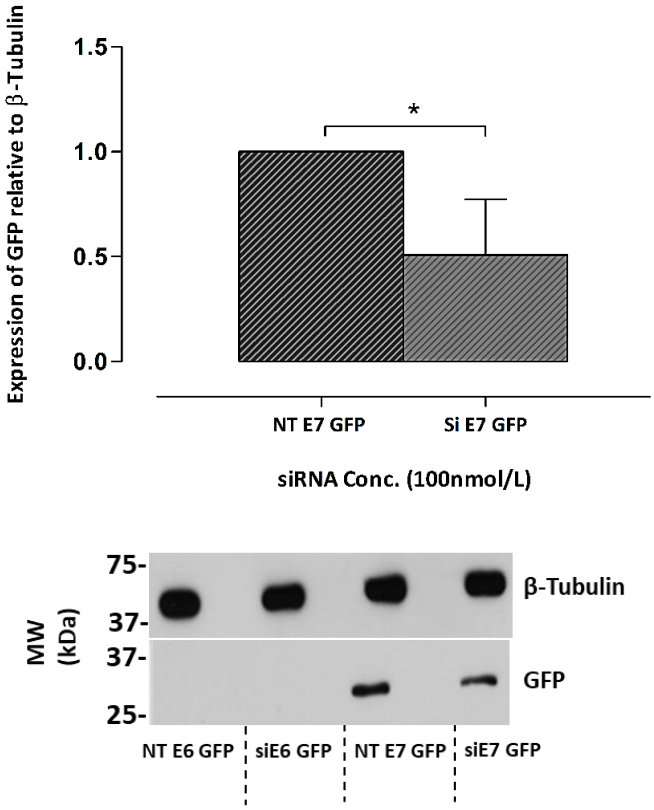

Figure 28. *Western blot analysis of GFP tagged CRPV E7 expression in COS-7 cells as a result of CRPV_E7 siRNA transfection. Although, β-tubulin bands of COS-7 cells expressing CRPV E6 GFP treated with siRNA can be observed in western blot, however CRPV E6_GFP protein bands could not be detected*

Western blot analysis revealed lower expression of CRPV E7_GFP protein in COS-7 cells (figure 28). However, expression of GFP tagged CRPV E6 protein could not be detected in western blot analysis although bands of reference gene (β-tubulin) appeared. In another attempt to detect CRPV E6 protein on western blot membrane, CRPV E6 gene was tagged with RFP label and cloned plasmid was transfected in COS-7 cells. However, antibody against RFP tag did not show any CRPV E6 protein bands in western blot although beta tubulin bands again appeared nevertheless.

Unverified antibodies or non-availability of antibodies altogether limits the use of western blot assay in a lot of protein detection or quantification studies [151]. This assay shows a unique approach in which tagging fluorescent labels with oncoproteins makes the detection of oncoproteins possible in western blot even in the absence of a valid antibody. After

siRNA- mediated knockdown of CRPV E7 gene, the decreased level of E7_GFP protein could be detected by using anti-GFP antibody against GFP tag attached previously to CRPV E7 gene. However, by using the same method, CRPV (E6_GFP) protein could not be detected because of some unknown reasons.

3.6.4 Fluorescence microscopy analysis

Spectral microscopy to analyse expression of GFP uptake in cells under an Inverted fluorescence microscope is a convenient and effective method for direct assessment of transfection of GFP labelled plasmids [152].

To determine changes in expressions of GFP fluorescence, after treating previously transfected COS-7 cells with GFP tagged E6 and E7 plasmids and later treating these cells with siRNAs against these oncogenes, GFP expressing COS-7 cells were analysed under an inverted fluorescence microscope (Zeiss Axiovert 100 M) attached with a high-intensity mercury lamp and a triple bandpass filter.

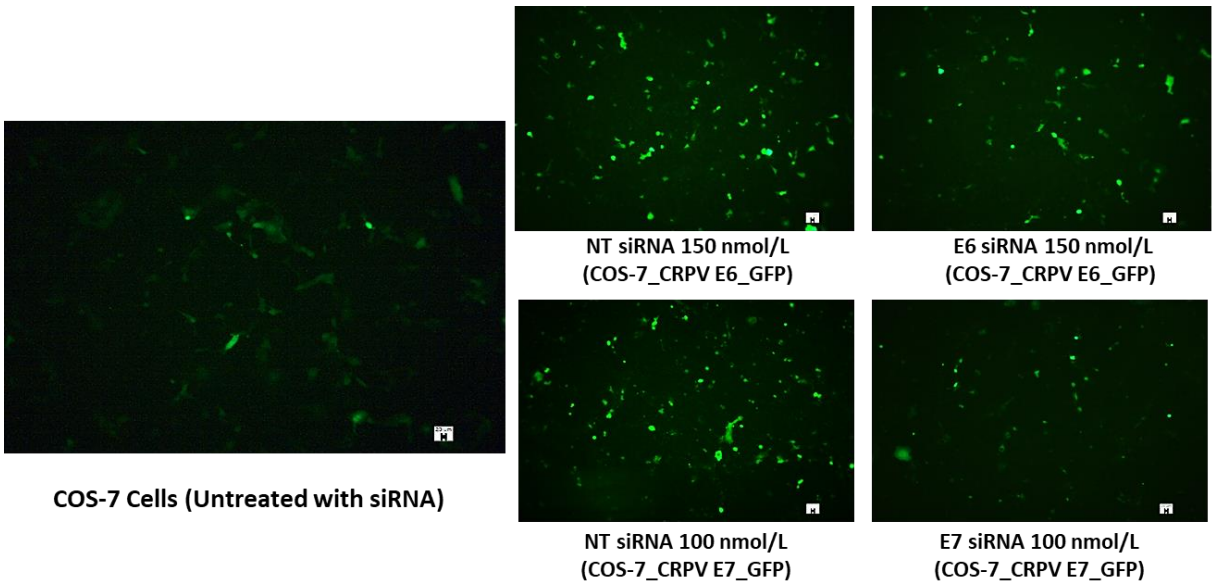

Figure 29. *Fluorescence microscopy image of GFP expression in COS-7 cells transfected by CRPV_E6 _GFP and CRPV_E7_GFP plasmids, followed by knockdown of CRPV E6 and E7 genes by CRPV E6 and E7 siRNA whereas non-target was used as control*

Visual inspection of GFP fluorescence exhibited lesser COS-7 cells expressing GFP post

CRPV E6 and CRPV E7 siRNA treatments compared to COS-7 cells treated with non-target siRNA as evident in figure 29.

3.6.5 Cytotoxicity analysis

MTT assay is the most extensively used method to analyse cell viability and cellular cytotoxicity in a wide range of experiments. This assay determines cells viability by evaluating activity of mitochondrial enzymes for example succinate dehydrogenase [153]. In this study, cellular cytotoxicity was measured by using MTT dye on COS-7 cells in order to witness effect of double transfection. COS-7 cells were first transfected by GFP tagged CRPV E6 and E7 plasmids in separate 6-well plates and post 24 h, transfected again by siRNA against CRPV E6 and CRPV E7 oncogenes, by using lipopolyplexes as transfecting reagents in both transfections. MTT assay was performed as described earlier and absorbance of viable cells induced formazan crystals was measured. All experiments were performed thrice.

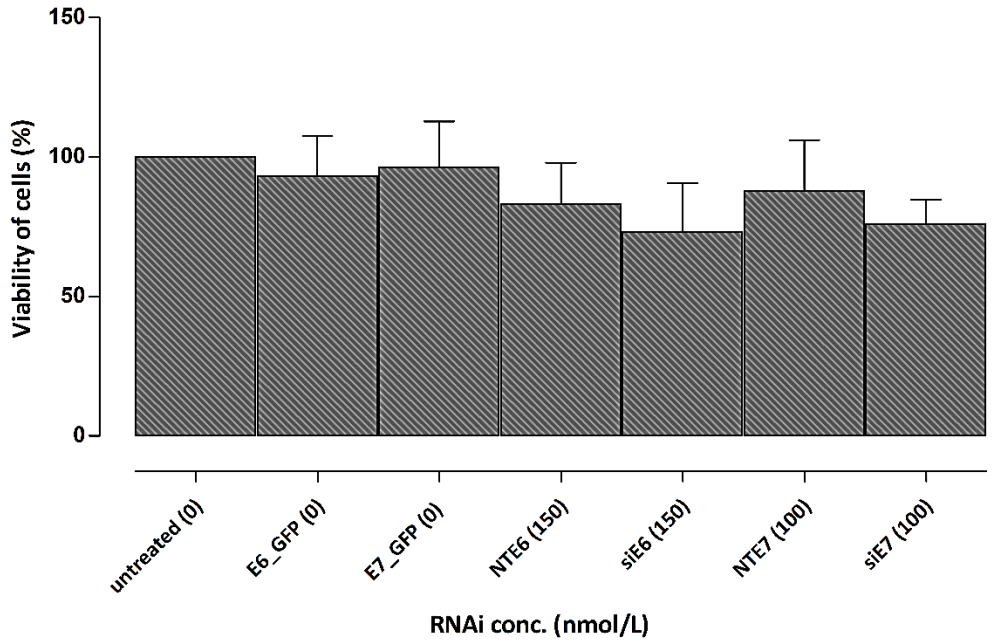

Figure 30. *Cell viability assay of untreated COS-7 cells, COS-7 cells after being transfected by GFP tagged CRPV E6 and E7 plasmids and COS-7 cells after re-transfection with siRNA loaded lipopolyplexes*

MTT assay outcome exhibited percentage of cell viability greater than 85 % in all samples which pronounced that lipopolyplexes are safe carriers for the transfer of GFP tagged CRPV E6 and E7 plasmids as well as CRPV E6 and E7 siRNA in COS-7 cells.

As COS-7 cells are "transiently" transfected by CRPV oncogenes and subsequently by siRNAs targeted against these oncogenes, these CRPV genes do not become a part of COS-7 genome and thus do not cause changes in intracellular machinery of COS-7 cells. With subsequent passages of COS-7 cells, these exogenous genes or siRNA is diluted out and finally disappears completely from the cells. So, cytotoxicity profile of lipopolyplexes as transfecting reagents in COS-7 cells (figure 30) shows only a slight decline in cell viability which is inevitable in case of a transfection process. This marginal bend in cell viability may be partially due to GFP which is assumed potentially cytotoxic for the cells [154] and partially due to lipopolyplexes, however the overall percentage of viable COS-7 cells is well above 80% as compared to untreated COS-7 cells.

Section IV

Limiting factors affecting gene therapy of CRPV carcinoma

3.7 Limiting factors affecting gene therapy of CRPV carcinoma

Tolerance to nutrient-starvation in cancer cells has been observed as a very important aspect of tumor development. A proper understanding of mechanisms of nutrient-independent growth of cancer cells seems to be essential in cancer therapy and research. In particular, aggressively growing tumors are more prone to experience nutrient-depletion because of uncontrollable proliferation as their rate of growth surpasses rate of nutrients providence. In these tumors, cell death regulatory proteins may be upregulated or downregulated in order to lend immunity to cancer cell from starvation induced apoptosis [155]. This study explains the relation between harsh environmental conditions and resultant changing gene expression profile of CRPV E6 and E7 oncogenes in VX2 carcinoma which is an aggressive and rapidly growing tumor. In molecular targeting therapy such as siRNA mediated gene therapy, the

dramatic changes in oncogenes expressions can lead to the failure of treatment. This oncogene expression profiling of CRPV E6 and E7 under harsh conditions highlight the potential limiting factors which can cause the cancer cells to deregulate the usual expression of those genes which are responsible for cell survival and proliferation and thus resulting in a different response to gene therapy than expected.

Serum starvation or nutrient depletion is experienced by the cancer cells *in vivo* during metastasis, invasion and tumor development as cancer cells require a lot of nutrients which can facilitate the rapid proliferation of tumors and when that does not happen, they become serum- depletion independent by overexpression or under-expression of various intracellular pathways [84].

3.7.1 Oncogenic profile shift under serum starvation

Various factors such as serum depletion or low nutrient supply can influence upon the expression of oncogenes involved in cellular functions and proliferation [156]. Cancer cells are capable of overcoming harsh micro-environment by modifications in their cellular signalling pathways resulting in alteration in survival-based gene expressions [84].

CRPV transformed VX2 cells express an invasive and resistant phenotype. Evaluation of serum-starvation induced effects on the expression of CRPV oncogenes such as E6 and E7 in VX2 cells may suggest the behavior of these oncogenes to promote cell survival and stimulate the cellular escape from nutrient-depletion induced apoptosis.

For this purpose, various serum starvation schemes were designed and applied on VX2 cells as described previously with close monitoring of CRPV E6 and E7 expression levels. Gene expression was analysed by RT qPCR.

Serum starvation setup (a)

Under serum starvation conditions as described previously in (2.5.1.1) CRPV E6 and E7 oncogenes exhibited dramatically huge expression levels in this particular experiment and their magnitude exceeded VX2 tumor tissue sample which was used as a positive control. In

order to affirm the accuracy of the RT qPCR results, three housekeeping genes, GAPDH, RPL32 and RPLPO were used.

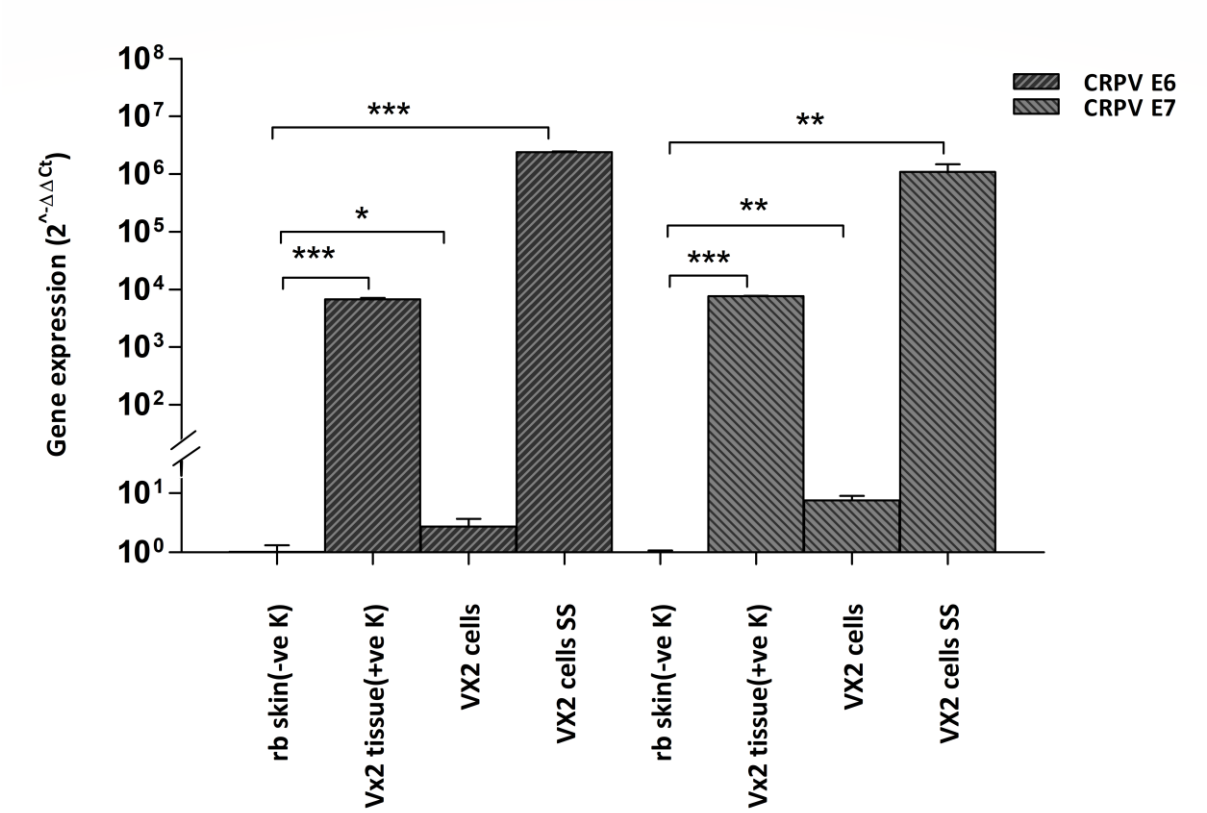

Figure 31. *RT qPCR analysis of E6 and E7 gene expression in VX2 cells grown in normal medium (10% FCS) versus serum starved medium. "VX2 cells SS" represent serum-starved cells whereas "VX2 cells" illustrate serum-fed cells*

This outcome shows that serum-starvation can lead to unexpected changes in transcript levels of oncogenes which are essential for cell survival and cancer growth in VX2 cells.

Serum starvation setup (b)

Expression of CRPV E6 gene in VX2 cells grown in normal medium (10% FCS) contrary to VX2 cells grown in serum free medium. VX2 cells grown in both growth conditions were trypsinized every day and passaged to the same respective medium in which they were growing, for a total period of seven days.

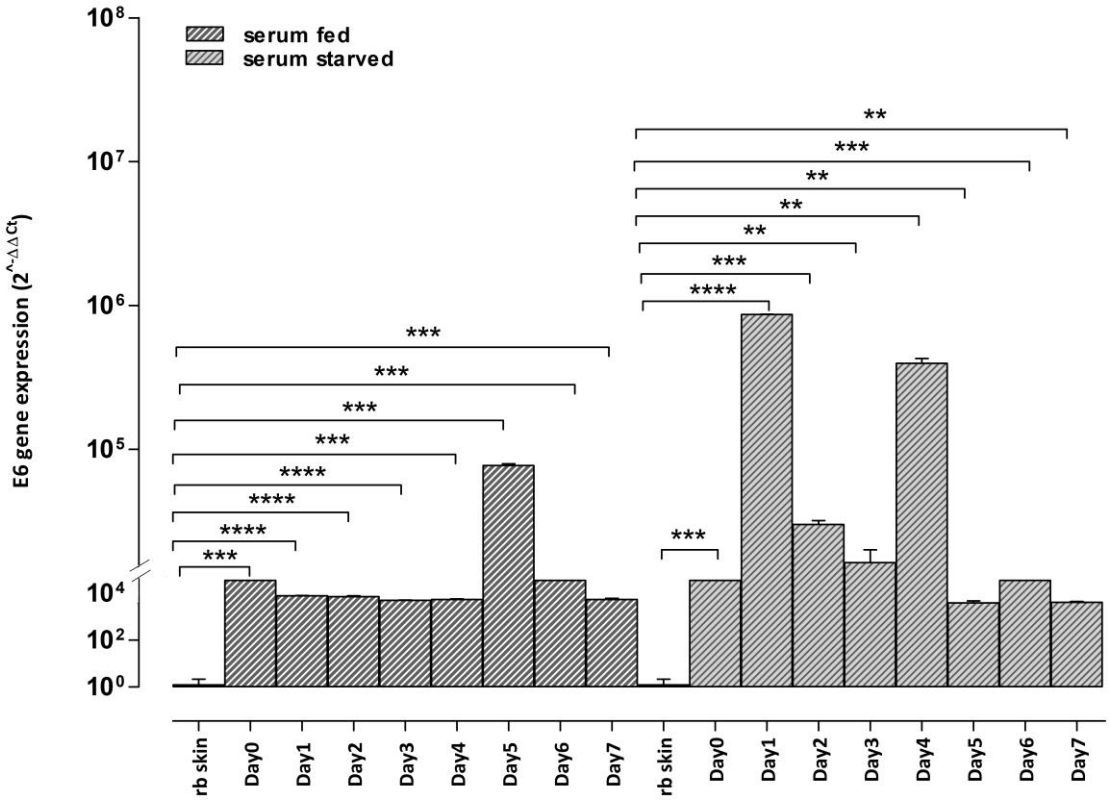

Figure 32. *Time course of E6 gene expression in VX2 cells treated with and without serum per day for seven days*

A daily account of CRPV E6 and E7 expression levels under serum-fed and serum-starved conditions of VX2 cells for seven consecutive days has been exhibited (figure 32). It is evident that most of the days, CRPV oncogene levels fluctuate mainly under serum-starved conditions towards higher end. In case of serum-fed VX2 cells, oncogenes levels fluctuated on a single day and remained more or less the same for other six days.

Serum starvation setup (c)

Another scheme to compare expression of CRPV E6 gene levels in serum-fed VX2 cells versus serum-starved VX2 cells per day basis and per week basis is shown below (figure 33).

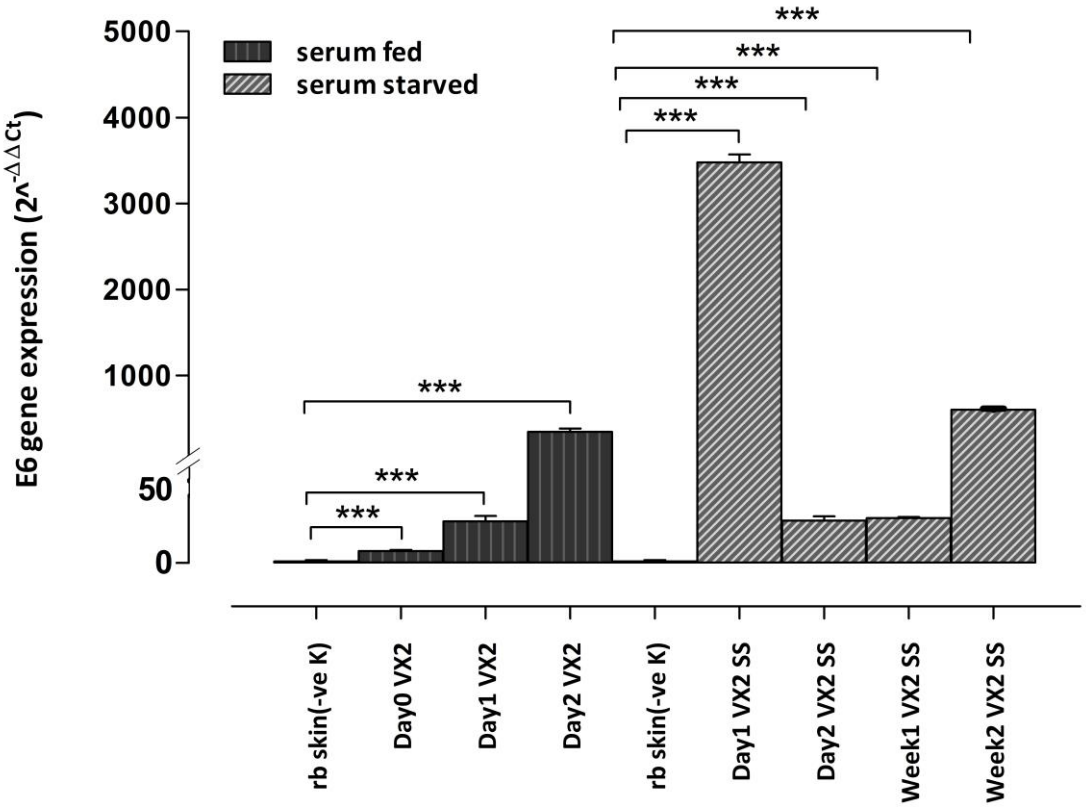

Figure 33. *RT qPCR analysis of E6 gene expression in VX2 cells grown in normal medium (10% FCS) and VX2 cells grown in serum free medium for one day, 2 days, one week and two weeks respectively*

VX2 cells grown in serum-starved medium for two consecutive days and in a separate setting, for two consecutive weeks, showed a persistent pattern of elevation of CRPV E6 oncogene level. The outcome of this serum-starvation plan is in accordance with the previous schemes which unanimously show an upregulation of CRPV oncogenes when VX2 cells experience desperate conditions of nutrients-depletion.

Serum starvation setup (d)

CRPV E6 and E7 gene expressions were analysed in VX2 cells grown in serum-fed and serum-starved medium for one week and during this period, medium on the cells was changed to fresh medium every day (figure 34).

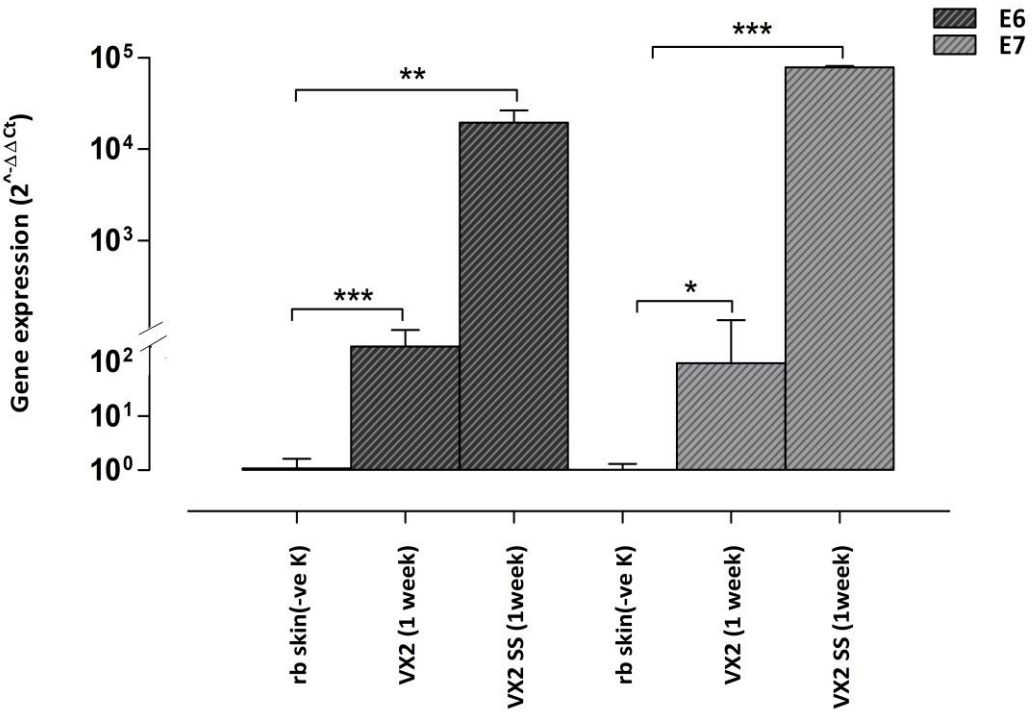

Figure 34. *Course of E6 and E7 gene expressions in VX2 cells during serum starved and serum-fed conditions after one week*

The data points to serum starvation induced upregulation of CRPV E6 and E7. The survival of VX2 cells under serum-starved conditions for a period of seven days points towards tolerance of these cells and ultimately resistance towards nutrient-depletion based apoptosis. The survival mechanism in full detail is yet unknown but upregulation of CRPV E6 and E7 certainly plays a role in VX2 cells survival.

3.7.2 Effect of Cisplatin exposure on VX2 cells

Exposure of chemotherapeutic agents such as cisplatin to cancer cells can exhibit upregulation of specific genes such as ABC (ATP-binding cassette) transporter which are involved in efflux of toxic substances such as cisplatin [157, 158]. Similarly, human ovarian cancer cells cisplatin exposure at a conc. of 40 µmol/L, for as short as 1 h, induced expression of ERCC-1 (excision repair cross-complementation group 1) which is involved in nucleotide excision repair [159].

To evaluate cellular effects of cisplatin on CRPV oncogenes, different concentrations of cisplatin were deployed and changes in the expression of CRPV E6 and E7 were monitored.

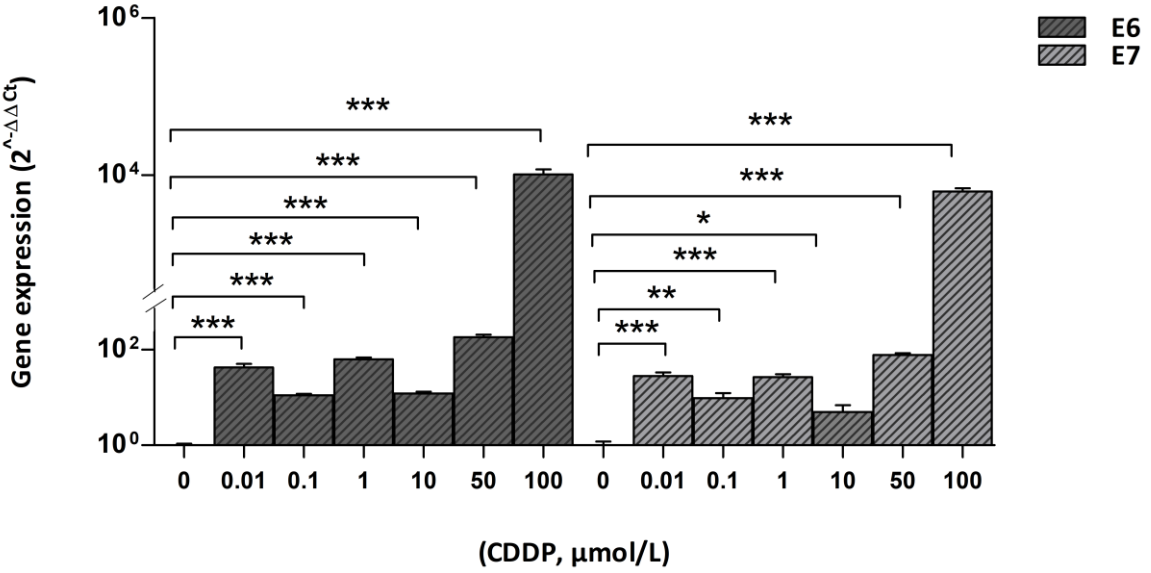

Figure 35. *VX2 cells treated with different concentrations of Cisplatin and their effect on the expression of CRPV E6 and E7 oncogenes*

Gene expressions of CRPV E6 and E7 were found to climb up many folds higher at a Cisplatin concentration of 100 µmol/L (figure 35). This data suggests that under extreme stress conditions, VX2 carcinoma has a tendency to become equally aggressive and activate cell survival pathways by upregulation of oncogenes which are responsible to keep tumor alive and proliferative.

At a Cisplatin concentration of 10 µmol/L, VX2 cells showed downregulation of oncogenes E6 and E7 (figure 35), which may be due to the fact that this concentration of Cisplatin is not too high for the cells to kick start their cellular survival machinery.

This delicacy of oncogenic shift under specific conditions may affect greatly on choosing the therapeutic options as they should be flexible to the changing character of oncogenic expression.

3.7.3 Effect of Radiation and Olaparib on VX2 Cells

Radiation induced transcriptional and translational changes are reported frequently depending upon the type of cell line [160]. VX2 cells were exposed to 40 µmol/L Olaparib followed by radiation (4Gy) and their effect was monitored for 24 h on CRPV E6 gene expression. As exposure to radiation causes DNA damage in cancer cells [161, 162], it could optimize the tumor inhibiting effect especially in the presence of radiosensitizers such as Olaparib.

RT qPCR analysis was deployed to analyse if there were changes in CRPV E6 expression when VX2 cells were exposed to Olaparib and subsequent radiation.

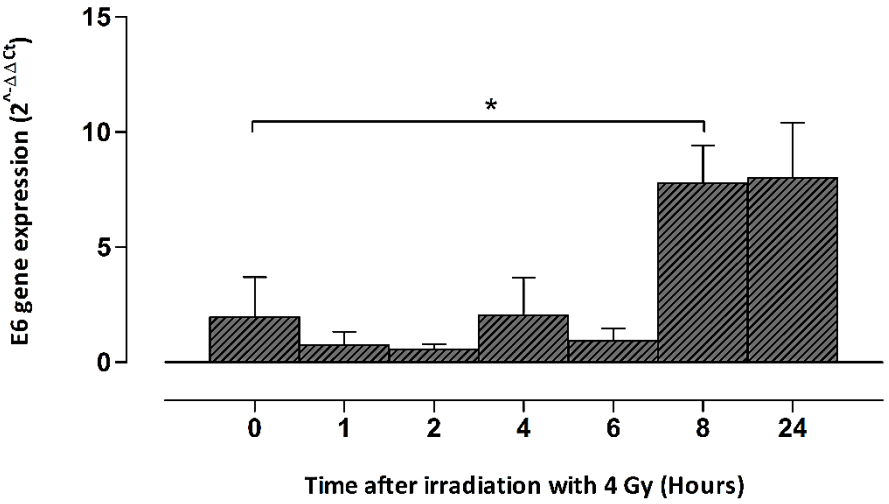

Figure 36. *Time course of VX2 cells treated with 40 µmol/L Olaparib followed by radiation (4 Gy) and their effect on CRPV E6 expression*

Above results of RT qPCR analysis show that exposure of VX2 cells to 40 µmol/L Olaparib and treatment with 4 Gy induced overexpression of CRPV E6 at 8 h and 24 h. After radiation therapy, cancer cells have an ability to stop cell cycle at specific checkpoints so that they can repair DNA damage. If tumor cells succeed in repairing DNA damage, they develop radiation resistance and can survive and proliferate [163].

Above data shows that after 8 hours to 24 hours, there is upregulation in CRPV E6 level which indicates that DNA repair has been achieved by VX2 cells and they started their proliferative machinery in order to replicate.

This result suggests that a close check on the expression level of CRPV E6 oncogene can reveal a lot of information about the cellular survival and proliferation at the end of a therapeutic application in CRPV carcinoma. It can also help to recognize the cell survival potential of cancer cells and to choose the best strategy regarding therapeutic window in order to eradicate cancer cells. In summary, all the stress inducing experiments including serum starvation, Cisplatin exposure and radiation therapy appear to affect CRPV E6 and E7 mRNA expression levels. Identification of behavior of oncogenes CRPV E6 and E7, under different treatment conditions can lead to effective approach for the treatment of CRPV cancers where caution should be maintained to not elicit aggressive upregulation of oncogenes while keeping the treatment threshold to the most effective range of therapeutic window.

3.7.4 Relation between oncogenic expression shift and gene therapy of CRPV induced VX2 carcinoma

To discover an *in vitro* treatment without a deep insight and comprehension of potential risks for the failure of its therapeutic application *in vivo* may cause serious consequences.

siRNA targeting therapy against oncogenes depends on a lot of factors such as expression levels of oncogenes, concentration of siRNA, transfection reagent efficiency and cytotoxicity etc. If there is too much fluctuation in oncogene expressions, such as in CRPV carcinoma, under certain conditions which are not fully known, it may be difficult to adjust siRNA concentration and consequently volume of transfection reagent to a level which has least cytotoxicity and efficient knockdown. The therapeutic approaches should also be lenient enough to ensure a persistent oncogenic expression profile and exert their anticancer effects to the maximum.

Part IV: Summary and outlook

4.1 Summary and Outlook

The VX2 carcinoma model from the New Zealand white rabbits serves as a suitable model system for human head and neck squamous cell carcinoma. Both cancer models share the common papilloma virus. HPV and CRPV both possess the oncogenes E2, E6 and E7. This thesis deals with one of the most vital fields of molecular targeting therapy, the siRNA induced gene silencing. An extensive literature review-based concept of parallel therapeutic options for HPV and CRPV induced carcinomas using siRNA has been addressed. Transfection of siRNA into cancer cells is very important for the successful RNAi mediated knockdown of oncogenes, therefore the pros and cons of using lipopolyplexes for transfecting VX2 cells and a model COS-7 cell line have been analysed.

The introduction part of this thesis comprises of an overview of some fundamental aspects of development of cancer. A brief insight into carcinogenicity of HPV and CRPV is discussed individually as well as together as both carcinomas originate from papilloma virus. Functional features of CRPV E6 and E7 oncogenes are described to highlight their role in the development of CRPV positive cancers. Certain attributes of these oncogenes which play a key role in immortalisation of rabbit keratinocytes or fibroblasts are listed. The importance of establishing an *in vitro* system to investigate the therapeutic options in CRPV induced carcinomas is emphasised by discussing limitations involved in evaluation of treatment strategies using *in vivo* models. The mode of action and efficacy of siRNA treatment in curing cancers is described by evidence-based research. The method of preparation of lipopolyplexes and their role as transfecting reagents for siRNA and plasmid delivery into cells is highlighted based on previously published data.

In materials and methods section, the preparation of lipopolyplexes, physicochemical properties, transfection efficiency and cytotoxicity evaluation in VX2 and COS-7 cells and the analytical methods here for are described. A detailed account of siRNA targeting against CRPV E6 and E7 oncogenes in VX2 cells and confirmation of downregulation of these oncogenes using various assay techniques is detailed. An analogous knockdown of CRPV E6 and E7 was carried out in a non CRPV cell line, COS-7, as an alternative approach, which is beneficial since a long-term *in vitro* culture of CRPV positive VX2 cells is not possible.

The *in vitro* studies on VX2 cells for the knockdown analysis of CRPV E6 and E7 genes was the focus of this project. A detailed behaviour of VX2 cells under different stress factors and resulting alteration in expression of oncogenes was documented. A novel method using flow cytometry was introduced to evaluate downregulation of CRPV E6 and E7 oncogenes in stable transfected COS-7 cells with GFP tagged plasmids. This method is a unique and precise way to determine knockdown of a gene as a result of siRNA treatment by detection of GFP signal alteration in treated and non-treated cells. A parallel study was performed on the cell suspension from VX2 tumor and the effect of siRNA treatment on downregulation of CRPV E6 and E7 oncogenes was analysed.

The results and discussion part of this thesis deals with the transfection efficiency of lipopolyplexes analysed by FACS and RT qPCR assays on VX2 and stable transfected COS-7 cells. Knockdown of CRPV E6 and E7 oncogenes in VX2 cells has been studied in detail and confirmed by RT qPCR, Western Blotting, FACS, MTT assay, Wound healing assay, Branching Morphogenesis Microscopy assay and xCELLigence TEER measurement assay. All these analytical evaluations showed downregulation of CRPV E6 and E7 oncogenes as a result of siRNA loaded lipopolyplex targeting. To get a deeper insight into the extent of efficacy of siRNA treatment on VX2 cells, CRPV E6 and E7 knockdown was carried out in a cell suspension prepared by trituration of raw VX2 tumor harvested from tumor bearing rabbits. siRNA mediated downregulation of CRPV E6 and E7 were analysed by RT qPCR. Another group of experimentation was designed by using a non CRPV cell line, COS-7. GFP tagged CRPV E6 and E7 plasmids were stably transfected in these cells. After detection of expression of GFP, COS-7 cells were transfected by siRNA against these CRPV oncogenes and the downregulation was analysed by FACS, RT qPCR, Western Blotting and MTT assay. Significant knockdown of CRPV E6 and E7 genes in COS-7 cells was noted which also confirmed the efficacy of lipopolyplex based siRNA treatment in exogenous settings. Next, the effect of different stress factors on the gene expression of CRPV E6 and E7 genes in VX2 cells was explored by creating various scenarios of serum starvation, radiation therapy in combination with olaparib and by cisplatin treatment. As a result, it has been suggested that a non-linear relationship exists between *in vitro* conditions and oncogene expression in VX2 cells. This can be taken as a basic cue to regulate cancer cell response to various treatments by also taking into consideration exposure to stress conditions in the cells which may alter cellular pathways and oncogene expression levels.

Collectively, this thesis identifies *in vitro* aspects which may influence the expression of

CRPV E6 and E7 oncogenes in CRPV induced carcinomas and knockdown of these genes by lipopolyplex based siRNA targeting. Various assays have confirmed the efficacy of siRNA against CRPV E6 and E7 oncogenes endogenously as well as exogenously. Investigations carried out in this work also confirm the transfection efficacy and safety of lipopolyplexes as transfecting reagents with the added benefit of being cost effective as compared to commercially available transfecting reagents.

This study can be used to investigate complexities involved in setting the magnitude of an *in vitro* treatment for translating it into an in *vivo* therapy as cancer cell lines behave in a non-linear fashion under different situations of therapeutic investigations and resultant stress. This prompts the need for a broader investigation of siRNA treatment and related induction of exaggerated expression of oncogenes by doing parallel studies on HPV positive cell lines. siRNA mediated silencing of CRPV E6 and E7 can be further investigated *in vivo* to observe the efficacy of this therapeutic approach in tumor bearing animals.

4.2 Zusammenfassung und Ausblick

Das VX2-Karzinommodell des neuseeländischen weißen Kaninchens dient als geeignetes Modell für das menschliche Plattenepithelkarzinom im Kopf-Hals-Bereich. Als Auslöser der Karzinome gelten u.a. Papillomaviren. Papillomaviren wie HPV und CRPV besitzen die Onkogene E2, E6 und E7. Die vorliegende Dissertation befasst sich mit einem der wichtigsten Bereiche der zielgerichteten Therapie, dem siRNA-induzierten Gen-Silencing. Die Erarbeitung eines Konzeptes, auf Basis bestehender Literatur, zur Behandlung von HPV- und CRPV-induzierten Karzinomen war Hauptbestandteil der dieser Arbeit. Die Transfektion von siRNA in Tumorzellen ist sehr wichtig für den erfolgreichen RNAi-vermittelten Knock-down der vorhandenen Onkogene. Die ausführliche Diskussion der Vor- und Nachteile eines Lipopolyplex vermittelten Gen Knock-downs in VX2- und COS-7-Zellen ist ebenfalls Gegenstand der hier dargestellten Untersuchungen.

Der einleitende Teil dieser Arbeit enthält einen Überblick über einige grundlegende Aspekte der Tumorgenese sowie eine kurze Darstellung der Kanzerogenität der Papillomaviren HPV und CRPV. Ebenso werden die funktionellen Eigenschaften der CRPV E6- und E7-Onkogene beschrieben, um ihre Rolle bei der Entwicklung von CRPV-positiven Tumoridentitäten zu verdeutlichen. Bestimmte Eigenschaften dieser Onkogene, welche eine Schlüsselrolle bei der Immortalisierung von Kaninchen-Keratinozyten oder -Fibroblasten spielen, werden dargestellt und diskutiert. Die Bedeutung der Etablierung eines *in-vitro*-Systems zur Untersuchung der therapeutischen Optionen bei CRPV-induzierten Karzinomen wird verdeutlicht. Die Wirkungsweise und Wirksamkeit der siRNA-Behandlung bei der Heilung von Tumorerkrankungen stellt die Möglichkeiten der evidenzbasierten Forschung dar. Die Herstellungsmethode von Lipopolyplexen und ihre Bedeutung als Transfektionsreagenzien für die siRNA- und Plasmidabgabe in Zellen ist ebenfalls Bestandteil dieses Kapitels.

Der Abschnitt Materialien und Methoden beschreibt die genaue Herstellung von Lipopolyplexen, die Messung der physikalisch-chemischen Eigenschaften, die Ermittlung der Transfektionseffizienzen und die Zytotoxizitätsbestimmung in VX2- und COS-7-Zellen. Eine detaillierte Darstellung der siRNA, die gegen die CRPV E6- und E7-Onkogene in VX2-Zellen gerichtet ist, und die Bestätigung der Herunterregulierung dieser Onkogene unter

Verwendung verschiedener Assay-Techniken ist ebenso Inhalt dieses Abschnitts. Ein Knock-down der Onkogene CRPV E6 und E7 wurde in einer Nicht-CRPV-Zelllinie, COS-7 als Modellzelllinie nachgewiesen. Der Grund für die Entwicklung dieser Modellzelllinie, mit stabil transfizierten CRPV E6 und E7, war, dass eine langfristige *in-vitro*-Kultur von CRPV-positiven VX2-Zellen nicht realisierbar ist. Die *in-vitro*-Studien an VX2-Zellen für die Knock-down Analyse der CRPV-Gene E6 und E7 standen im Mittelpunkt dieses Projekts. Es wurde detailliert das Verhalten von VX2-Zellen unter verschiedenen Stressfaktoren und die daraus resultierende Veränderung der Expression von Onkogenen untersucht. Eine neue Methode, unter Verwendung der Durchflusszytometrie, konnte etabliert werden um die Herunterregulierung der CRPV E6- und E7-Onkogene in stabil transfizierten COS-7-Zellen mit GFP-getaggten Plasmiden zu analysieren. Diese Methode ist eine neuartige und äußerst präzise Methode zur Bestimmung des Knock-downs eines Gens als Folge einer siRNA-Behandlung. Es kommt hierbei zu einer GFP-Signalveränderung in behandelten und unbehandelten Zellen. Eine Parallelstudie wurde an einer Zellsuspension des VX2-Tumors durchgeführt um die Wirkung der siRNA-Behandlung auf die Downregulation der CRPV-Onkogene E6 und E7 zu charakterisieren.

Der Ergebnis- und Diskussionsteil dieser Arbeit befasst sich mit der Transfektionseffizienz von Lipopolyplexen, die man mittels FACS- und RT-qPCR-Assays an VX2 an stabil transfizierten COS-7-Zellen analysiert hat. Eine Bestätigung der Knock-downs von CRPV E6- und E7-Onkogenen in VX2-Zellen erfolgte mit Hilfe moderner molekularbiologischer Arbeitstechniken wie RT-qPCR, Western Blotting, FACS, MTT-Assay, Wundheilungsassay, Branching Morphogenesis Microscopy Assay und das xCELLigence TEER Measurement Assay. All diese analytischen Auswertungen zeigten eine Herunterregulierung der CRPV E6- und E7-Onkogene als Ergebnis der Behandlung mit siRNA-beladenen Lipopolyplexen. Um einen tieferen Einblick in das Ausmaß der Wirksamkeit der siRNA-Behandlung von VX2-Zellen zu erhalten, wurde der CRPV E6- und E7-Knockdown in einer Zellsuspension durchgeführt. Dies fand durch Isolierung der Zellen eines VX2-Tumors, von tumortragenden Kaninchen, statt. Die siRNA-vermittelte Herunterregulierung von CRPV E6 und E7 wurde durch RT-qPCR analysiert. Weiterführende Experimente wurden an der Modellzelllinie COS-7 durchgeführt. Die stabile Transfektion dieser Modellzellen erfolgte mit GFP-markierten CRPV E6- und E7-Plasmiden. Nach dem Nachweis der Expression von GFP wurden COS-7-Zellen mit siRNA gegen CRPV-Onkogene transfiziert. Eine Analyse des Knock-downs erfolgte mit Hilfe von

FACS, RT-qPCR, Western Blotting und MTT-Assay. Der Knock-down CRPV E6- und E7-Gene in COS-7-Zellen war signifikant und bestätigte auch die Wirksamkeit einer Lipopolyplex-vermittelten siRNA-Behandlung. Als nächstes erfolgte die Untersuchung der Wirkung verschiedener Stressfaktoren auf die Genexpression der CRPV E6- und E7-Gene in VX2-Zellen. Hierbei wurden verschiedene Szenarien wie Serumverhungern, Strahlentherapie in Kombination mit Olaparib und durch Cisplatin-Behandlung in die Untersuchungen einbezogen. Als Ergebnis wurde vermutet, dass eine nicht-lineare Beziehung zwischen *in vitro*-Bedingungen und der Onkogenexpression in VX2-Zellen besteht. Dies kann als ein Anhaltspunkt für die Regulierung von Tumorzellen auf verschiedene Behandlungen angesehen werden. Stressbedingungen verursachen bei Tumorzellen eine Änderung des Onkogenexpressionsnveaus.

Zusammenfassend behandelt diese Arbeit *in-vitro* Aspekte, die die Expression der CRPV E6- und E7-Onkogene in CRPV-induzierten Karzinomen und den Knock-down dieser Gene durch Lipopolyplex-basierte siRNA-Theapie beeinflussen können. Verschiedene Assays haben die Wirksamkeit der siRNA gegen die CRPV-Onkogene E6 und E7 sowohl endogen als auch exogen bestätigt. Die in dieser Arbeit durchgeführten Untersuchungen bestätigen auch die Transfektionswirksamkeit und -sicherheit von Lipopolyplexen mit dem zusätzlichen Vorteil, dass sie im Vergleich zu kommerziell erhältlichen Transfektionsreagenzien kostengünstig sind.
Diese Studie kann verwendet werden, um die Komplexität zu untersuchen, die mit der Umsetzung einer *in-vitro* Behandlung zu einer *in-vivo* Therapie einhergeht. Der Grund hierfür ist das nicht-lineare Verhalten von Tumorzellen unter verschiedenen therapeutischen Untersuchungen und Stressbedingungen. Daraus ergibt sich die Notwendigkeit einer breiteren Untersuchung der siRNA-Behandlung und der damit verbundenen Induktion einer erhöhten Expression von Onkogenen durch parallele Studien an HPV-positiven Zelllinien. Der siRNA-vermittelte Knock-down von CRPV E6 und E7 kann *in-vivo* weiter untersucht werden, um die Wirksamkeit dieses therapeutischen Ansatzes bei tumortragenden Tieren zu beobachten.

Part V: Appendix

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5.2 List of abbreviations

APS	Ammoniumpersulphate
BSA	Bovine serum albumin
bp	Base Pairs
bPEI	Branched PEI
CI	Cell index
CCD	Charged Coupled Device
CLSM	Confocal Laser Scanning Microscopy
CRPV	Cottontail Rabbit Papilloma Virus
DAPI	4',6-diamidino-2-phenylindole
DDC	DOPE, DPPC, Cholesterol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DPPC	Dipalmitoylphosphatidylcholine
dsiRNA	Dicer Substrate Short Interfering Ribonucleic Acid
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminetetraacetic acid
EGF	Endothelial Growth Factor
EGFP	Enhanced Green Fluorescence Protein

FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescence Protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	Human Papilloma Virus
HNSCC	Human Head and Neck Squamous Cell Carcinoma
HRP	Horseradish Peroxidase
kDa	Kilodalton
LF	Lipofectamine 2000™
lPEI	Linear Polyethylenimine
LPP	Lipopolyplexes
MW	Molecular, Cellular and Developmental Biology (medium)
MI	Mechanical Index
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NP40	Nonidet P 40 substitute
PBS	Phosphate Buffered Saline
PCI	Photochemical Internalisation
PDI	Polydispersity Index
pDMAEMA	Poly (2-dimethylaminoethyl methacrylate)
pDNA	Plasmid Deoxyribonucleic Acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PLL	Poly-L-lysine
PP	Polyplex
RISC	Ribonucleic Acid Induced Silencing Complex
RLU	Relative Luminescence Units
RNA	Ribonucleic Acid
SiRNA	Ribonucleic Acid Interference
rpm	Revolutions per minute
RT	Room Temperature
SD	Standard Deviation
SDS	Sodium-Dodecyl-Sulphate
SEM	Scanning Electron Microscope
siCtrl	Control Short Interfering Ribonucleic Acid
siRNA	Short Interfering Ribonucleic Acid
SOC	Super Optimal Broth with Catabolite Repression
TBE	Tris(hydroxymethyl)aminomethane/Borate/ Ethylenediaminetetraacetic acid
TEER	Transepithelial electrical resistance
TEMED	N, N, N', N'-tetramethyl ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UEGT	Ultrasound Enhanced Gene Transfer
VEGF	Vascular Endothelial Growth Factor

5.3 Research Output

Imran Tariq, Muhammad Yasir Ali, Harshavardhan Janga, Sajid Ali, Muhammad Umair Amin, Ghazala Ambreen, **Uzma Ali,** Shashank Reddy Pinnapireddy, Jens Schäfer and Udo Bakowsky; Downregulation of MDR 1 gene contributes to tyrosine kinase inhibitor induced apoptosis and inhibition in tumor metastasis: A gravity to space investigation. *Manuscript accepted.*

Ghazala Ambreen, Imran Tariq, Lili Duse, **Uzma Ali**, Sajid Ali, Shashank R. Pinnapireddy, Michael Bette, Udo Bakowsky, Robert Mandic; Sensitivity of papilloma virus associated cell lines to photodynamic therapy with curcumin loaded liposomes. *Manuscript submitted.*

Uzma Ali, Ghazala Ambreen, Shashank R. Pinnapireddy, Imran Tariq, Michael Bette, Udo Bakowsky, Robert Mandic; siRNA-mediated endogenous and exogenous downregulation of CRPV E6 and E7 oncogenes. *Manuscript in preparation.*

Uzma Ali, Shashank R. Pinnapireddy, Ghazala Ambreen, Michael Bette, Robert Mandic, Udo Bakowsky; A comprehensive recount of transfection efficiency and cytotoxicity of lipopolyplexes in different cell lines. *Manuscript in preparation.*

Uzma Ali, Shashank R. Pinnapireddy, Ghazala Ambreen, Michael Bette, Robert Mandic, Udo Bakowsky; Oncogenic behavior of CRPV positive cells under different cellular stress stimuli. *Manuscript in preparation.*

Ghazala Ambreen, **Uzma Ali,** Shashank R. Pinnapireddy, Michael Bette, Udo Bakowsky, Robert Mandic; Establishment and characterization of CRPV based cell systems as an alternative approach to study papilloma virus associated HNSCC. *Manuscript in preparation.*

5.4 Presentations

Establishment and characterization of a VX2 carcinoma derived rabbit cell line for the study of papilloma virus associated head and neck cancer- Oral Presentation: **Uzma Ali**, Ghazala Ambreen, Shashank Reddy Pinnapireddy, Eva Mohr, Boris A. Stuck, Udo Bakowsky, Michael Bette, Robert Mandic, (May 29th-June 1st 2019) "90th Annual Meeting of the German Society of Oto-Rhino-Laryngology, Head and Neck Surgery" Berlin, Germany

siRNA-Induced mRNA knockdown of HNSCC associated CRPV E6 and E7 Oncogenes using Lipopolyplexes as Transfecting Reagents- Oral Presentation: **Uzma Ali**, Ghazala Ambreen, Shashank Reddy Pinnapireddy, Eva Mohr, Boris A. Stuck, Udo Bakowsky, Michael Bette, Robert Mandic, (7-8 March, 2019) Functional biomaterials and release of nucleic acid drugs" 23nd Annual meeting7/8, CRS, German Local Chapter, Leipzig, Germany

Targeting CRPV E6 and E7 oncoproteins using low toxicity LPP packaged siRNA as a paradigm for the treatment of human HPV-associated HNSCC- Poster Presentation: Ghazala Ambreen, **Uzma Ali**, Shashank Reddy Pinnapireddy, Eva Mohr, Boris A. Stuck, Udo Bakowsky, Michael Bette, Robert Mandic, (18-19 Jan, 2019) Precision Oncology and Personalized medicine for Head & Neck Cancer" Heidelberg, Germany

The VX2 carcinoma of the rabbit as a suitable animal model for papilloma virus associated head and neck squamous cell carcinomas- Poster Presentation: Robert Mandic, Udo Bakowsky, **Uzma Ali**, Ghazala Ambreen, Nora Franke, André Marquardt, Elisabeth Mack, Boris A. Stuck, Michael Bette, (25-27 Jan 2018) 2nd International Symposium on "Tumour-Host interaction in H&N Cancer - 3rd International symposium on HPV Infection in Head & Neck Cancer", Essen, Germany

In Vitro Phenotyping and Oncogenicity Evaluation of VX2- Poster Presentation: **Uzma Ali**, Ghazala Ambreen, Shashank Reddy Pinnapireddy, Udo Bakowsky, (2-3 March 2017) 21st Annual meeting Controlled Release Society "Future Trends in Nanomedicine" Marburg, Germany

5.5 Curriculum Vitae

PERSONAL DETAILS

Name	Uzma Ali
Date of Birth	1st April 1981
Place of Birth	Jhang, Pakistan
Marital Status	Married
Nationality	Pakistani
Email	uzmayali@yahoo.com

EDUCATION AND TRAINING

April 2016 - Present	Doctoral Researcher Dept. of Pharmaceutics and Biopharmaceutics, University of Marburg, Marburg (Germany)
Oct 2007 – Sep 2010	Master of Pharmaceutics University of Sargodha, Sargodha (Pakistan)
June 2007 - July 2007	Internship Mega Pharma Ltd, Lahore (Pakistan)
Oct 2003 - Oct 2007	Bachelor of Pharmacy (B. Pharmacy) Bahauddin Zakariya University, Multan (Pakistan)

SCHOOLING

Mar 2000 - Apr 2002	Intermediate Education (FSc. Pre-Medical) Board of Intermediate and Secondary Education, Faisalabad (Pakistan)
Mar 1996 - Apr 1998	Secondary Education (Matriculation Science) Board of Intermediate and Secondary Education, Faisalabad (Pakistan)

PROFESSIONAL EXPERIENCE

Apr 2010 – Dec 2014	Lecturer, (Pharmaceutical Technology, Industrial Pharmacy, Pharmaceutical Preparations and Instrumental Techniques at graduate level, practical lab work, conducting seminars) Department of Pharmacy, The University of Faisalabad, Faisalabad (Pakistan)

Nov 2007 - Jun 2008	Production Pharmacist, (Responsible for generation of daily production report & IPC of tablet, capsule & suspension section & maintenance of batch production records) Pharmix Industries Pvt Ltd, Lahore, Pakistan

RESEARCH FOCUS

- Composite polymer-liposome gene delivery systems
- Cell culture and cloning
- Characterization and validation of antibodies directed against oncogenes
- siRNA designing and validation against specific oncogenes
- Molecular biology of oncogenic markers and regulatory pathways

SKILLS

- Microbiological analyses
- Cell culture
- Cell based assays (in situ Hybridization, Immunohistochemistry)
- Flow cytometry
- qPCR and End Point PCR
- Western Blot
- xCELLigence Real Time Cell Analysis
- Data processing (MS Office), Biostatistics (Graphpad)
  Flowcytometry Analysis (Denovo FCS Express, Flowing software)
  Quantification of Bioimages (Imagej software, WIMasis image analysis)

MEMBERSHIPS

- American Association of Pharmaceutical Sciences (AAPS), USA
- The Marburg University Research Academy (MARA), Philipps-University Marburg, Germany